The interstitial cells of the urinary bladder

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Promotor
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Copromotor
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Voor mijn ouders, zonder hun steun zou
dit alles nooit mogelijk geweest zijn.

Contents

Chapter 1

General introduction, aim and structure of the thesis

Bladder function and pathology of the overactive bladder syndrome (OAB)

The function of the bladder seems to be rather simple. Urine is transported by the ureters towards the bladder to be stored. Once the bladder gets stretched to a certain degree of bladder filling, the brain gets a signal that the bladder needs to empty [1]. In order to void, the bladder wall is equipped with muscle fibres (detrusor muscle). Emptying the bladder is more complex than one would expect. While the detrusor muscle contracts, the sphincter around the urethra, has to relax. This mechanism is controlled at several different levels, in the brainstem, spine and in ganglia around the bladder [2].

Micturition is the result of a complicated signalling pathway, involving brainstem, spinal cord, and a signalling mechanism in the urinary bladder itself [2]. Especially our understanding of the afferent signalling pathway from the bladder to the central nervous system is still scanty. It now becomes apparent that the urothelium is involved in the micturition process by playing an important role in the afferent signalling [3]. The urothelium is not a passive barrier between urine and detrusor muscle alone; it is a highly specialized structure involved in antigen presentation, modulation of the micturition, metabolic secretion, inflammatory regulation, and sensory afferent functioning [3, 4]. The urothelium can respond to thermal, mechanical, and chemical stimuli and it acts as a transducer, releasing chemicals that target adjacent bladder cells, interstitial cells and sensory neurons [3, 4].

Control of the urinary bladder is of considerable clinical importance and has been the focus of a great deal of attention from both clinicians and basic scientists. Over the past century, experimental work on human bladder function has focused primarily on cystometric *in vivo* investigations and cellular, molecular and morphological studies of biopsy material. Because experiments in humans and on human tissue rises legal, moral and ethical dilemmas, such studies are inherently limited. For example, it is not possible to study the cellular processes experimentally in the CNS that control micturition in humans. Also it also is becoming extremely difficult to acquire significant amounts of bladder tissue from healthy normal subjects for investigation of cellular and physiological events. Thus, basic science has resorted to using tissue from animal models to gain insight into the principles governing integrated control of the lower urinary tract and its cellular components.

In normal subjects, bladder sensation increases as the bladder fills [5]. In patients with OAB, these sensations can be excessive, more frequent, and lead to urgency (the sudden and compelling desire to void that cannot be postponed) and urgency incontinence (inability to keep urine in the bladder). OAB is a symptom complex that comprises storage symptoms of urgency with or without urgency incontinence, frequency (voiding more often than 8 times in a 24-hour period) and nocturia [6]. These symptoms are suggestive for but not of diagnostic value for detrusor overactivity (urodynamically demonstrable involuntary bladder contractions) if there is no proven infection or other obvious pathology. OAB can, therefore, clearly distinct from urodynamically

proven detrusor overactivity, although the majority of people with OAB are thought to have this underlying diagnosis.

The prevalence of OAB increases with age [7]. From the epidemiological studies conducted to date, it can be concluded that of those patients with OAB, approximately one-third are troubled by incontinence (OAB 'wet') and two-thirds are not (OAB 'dry') [8, 9]. More recent data from the EPIC (European Prospective Investigation into Cancer and Nutrition) study suggest that the prevalence of OAB symptoms (using the 2002 International Continence Society (ICS) definition [10]) is close to 12% in the community, and of these sufferers, approximately 50% experience significant bother from their symptoms [11]. For the majority of patients, the patient history, physical examination, self-reported questionnaires and urine-analysis are sufficient to establish a working diagnosis of OAB. The majority of patients with OAB can be treated successfully with a combination of behavioural and pharmacological treatment. Behavioural treatment consists of bladder training, timed voiding, dietary modification, pelvic floor muscle exercises and behavioural modification, such as reduced intake of alcohol, caffeine, fizzy drinks and total fluid consumption. Burgio *et al.* have shown that the combination of behavioural and pharmacological therapy is more effective than either therapy alone [12].

The activity of the bladder during the filling phase is central to the origins of sensation [13]. Therefore, it seems obvious that the physiology of the filling phase and the cells responsible for this sensation should be examined to understand the causes of urgency and urgency incontinence. It is still widely thought that the human bladder is not active during the filling phase. Furthermore, it has become generally accepted that any detrusor activity during filling is pathological. However, this is not actually true. There is a body of evidence indicating that the bladders of healthy young subjects are capable of generating phasic increases in pressure during the filling phase [13]. The original observations of Mosso and Pellacani on nonrhythmic contractions in the human bladder before voiding were made more than 120 years ago [14]. In animals, the situation is clearer. It has been known for over a century that the bladder is active during filling: again, the original observations of this phenomenon in cats were made already in 1892, by Sherrington [15]. Sherrington also showed that this phasic activity originated in the absence of any CNS input and occurred when the bladder was removed and maintained *in vitro* [15]. Therefore a plausible conclusion is that this autonomous activity is inherent property of the bladder wall.

There are two distinct patterns of detrusor contractions: one associated with the overall contractions of the detrusor similar to the micturition contraction and the other generating complex phasic activity [13]. The different patterns are likely to be originated in different generative systems. In order to understand these mechanisms it is necessary to identify the cell types which play a role in both these systems. It is hypothesised that the autonomous contractions are responsible for the bladder sensation. If this indeed is the case, then an

upregulation of these autonomous contractions may result in increased sensations which in turn may be responsible for the OAB syndrome [13]. Therefore it is of utmost importance to know which cell types play key roles in this mechanism and how we can interfere pharmacologically with this mechanism.

Autonomous activity in the isolated bladder increases as the bladder is filled [13]. This suggest the possibility of 'local reflex' mechanisms active in the bladder wall that may be involved in some way in modulating detrusor activity [13]. The components of such a reflex are unknown. One possibility involves sensory nerves. Sensory nerve endings lie in the suburothelial spaces, and in many tissues these axons produce collaterals that remain in the tissue [16]. Such a micro anatomical arrangement was first described by Bayliss in 1901 to account for local vasodilator reflexes in the skin [17]. Activation of the afferent fibres results in antidromic activation of the collaterals, which then have their effect on the tissue. This local axonal reflex has been suspected in many other tissues, including the bladder (see Maggi and Meli for a review) [18]. However it has been suggested that other components should also be considered. It was suggested recently that interstitial cells in the suburothelial space might serve some form of sensory function [13]. It is not known how these cells contribute to sensation and how this mechanism may operate physiologically, but they could play a role in volume responses.

When the complex mechanism of filling and emptying the bladder becomes disordered, several functional complaints arise. The patients complain of urgency, frequency and incontinence. The underlying pathophysiology of OAB is not entirely understood. OAB has been attributed to neurogenic factors resulting in diminished inhibition of the micturition reflex or myogenic factors affecting the detrusor muscle itself [19]. A more recent hypothesis emphasizes the sensory role of the epithelium in response to bladder wall distension or epithelial or subepithelial sensitivity to multiple neurotransmitters including acetylcholine [20]. The autonomous bladder hypothesis described above states that the detrusor has a modular built-up [13]. Each module is defined by the area supplied by individual intramural bladder ganglia, or by a node of interstitial cells, which collectively are termed the myovesical plexus [13]. There can be synchronization of activity between modules. Activity could be propagated through the intramural nerve or interstitial cell networks, or by direct communication between muscle cells. This theory suggests that, during normal bladder filling, there is autonomous activity with non-micturition contractions and phasic sensory discharge [13]. These basic mechanisms can become modified in pathological conditions, causing a shift in balance either by excessive excitatory inputs or failure of inhibiting inputs, leading to inappropriate augmentation of autonomous activity and predisposing to detrusor overactivity [21]. Furthermore, any factor that enhances communication between modules will predispose to detrusor overactivity. Therefore a cell type

which has a crucial role in the autonomous bladder hypothesis is the interstitial cell (IC) [22].

History of ICs

In 1893 Cajal described a novel cell type in the gut [23]. He named these cells the interstitial cells, and they are presently known as the interstitial cells of Cajal (ICC). It can be found in the text books that these cells play an important role in gastrointestinal motility [24]. Based on morphological and in vitro physiological studies, ICC are believed to be pacemaker cells generating slow waves to serve as mediators in neural transmission and may also act as mechanoreceptors [24]. Thuneberg (1982) hypothesised that interstitial cells of Cajal in the gut act as pacemaker cells for gastrointestinal motor activity [22]. The ICC generate electrical slow waves, which passively transmit to gastrointestinal smooth muscle, they mediate cholinergic and nitrergic neurotransmission, they set the smooth muscle membrane potential and membrane gradient, and they also appear to be involved in mechano-transduction [24]. The current definition of a gastrointestinal ICC requires the surface expression of c-Kit (a cytokine receptor kind of protein) on a cell type that has at least two processes [25]. At the electron microscopy level, characteristics of ICC include the presence of intermediate filaments, numerous mitochondria and gap junctions [25].

Recognition of common principles of ICC organization (confinement to specific locations in relation to smooth muscle layers; formation of extensive cellular networks through tight coupling of overlapping thin processes; innervation patterns; characteristic patterns of contact with smooth muscle cells) and ultrastructure (myoid features: basal lamina, caveolae, rich in sER and mitochondria, often prominent filament bundles and dense bands/ bodies) has allowed Thuneberg *et al.* to identify ICC in the gastointestinal (GI) musculature [22]. However, variation in organization and ultrastructure is significant, between species and regions of the GI tract [26].

More recently ICs with many of the morphological and electrical features of ICCs have been discovered in other organs [27]. These cells have been identified in the ureter, bladder, urethra, vas deferens, fallopian tube, blood vessels, lymphatics, pancreas, prostate, penis, mammary gland, and myocardium [27]. In the renal pelvis and proximal ureter ICs have been identified and it has been demonstrated that these cells contribute to peristaltic contractions [28]. Cells with similar morphological characteristics have been found in the wall of blood vessels [29]. These vascular ICs demonstrated rhythmical Ca^{2+} oscillations associated with membrane depolarisations, which suggests that vascular ICs may act as pacemakers for smooth muscle cells [29]. Besides the putative pacemaker function, ICs might have other physiological roles, depending on tissue type (e.g. intercellular signalling, immune surveillance, steroid sensors) [30].

In 1996, Smet *et al.* characterised a cell type in the bladder wall of the human and the guinea pig which he characterised it as being stellate and spider-like in appearance with extended long cytoplasmic processes with extensive dendritic arborizations [31]. These cells responded to nitric oxide with a rise of cGMP [31]. This represents the potential physiological targets of neuronally released nitric oxide in the bladder, since activation of soluble guanylate cyclase, and a consequent rise in intracellular cGMP, mediate many of the effects of this transmitter [31]. Smet *et al.* called this cell-type an IC [31]. It was immediately recognized that these cells bore a striking resemblance to the ICC in the digestive tract [31].

ICs markers and function

The definition of ICs in the bladder is not straightforward. The morphology of these cells is similar to the ICC; however, they have also features in common with myofibroblasts. Both ICC and myofibroblasts are specialized fibroblasts [32]. The similarity in morphology of ICs with these other cells has resulted in the presently existing confusion about what are precisely ICs in the bladder, because in the literature on bladder physiology all four terms are used to indicate these cells.

Fibroblasts and myofibroblasts are characterized by several features: spindle- or stellate-cell morphology, a palely eosinophilic cytoplasm, and an abundant pericellular matrix [33]. The immunophenotype of myofibroblasts is: vimentin positive, alpha-smooth-muscle actin positive, smooth-muscle myosin negative, non-muscle myosin positive, virtually no desmin and fibronectin positive [33]. Ultrastructurally the myofibroblast has a prominent rough endoplasmic reticulum, a Golgi apparatus producing collagen secretion granules, peripheral myofilaments, fibronexus junctions and gap junctions [33, 34]. These features are similarly found in ICs in the bladder [35]. Therefore, clear-cut criteria for distinguishing between the different cell types are not available [35]. It has been suggested that these cells belong to the same population of cells of mesenchymal origin, which are adapted to special functional needs in their own micro-environment [35]. This latter suggestion appears to be especially true for the ICs in the bladder wall, as many different subpopulations of these cells have already been observed.

Phasic changes in pressure occur in bladders which are not associated with micturition [36]. Spontaneous intravesical pressure changes can be recorded from bladders *in vitro* or bladders *in vivo* isolated from the central nervous system suggesting that the bladder itself is capable of this autonomous activity [36]. Experiments using isolated cells and muscle strips indicate that the smooth muscle cells can generate spontaneous activity [37]. The bladder consists of a complex organisation of structures within the wall involving active elements including pacemaker regions, conductive pathways and contractile units in conjunction with regions of inactivity and passive stretch [38].

Hashitani points out that ICs in the bladder are not a simple analogy of ICCs in the gut, which act as electrical pacemaker, but represent an example of cell-type specific variability amongst tissues which is accounted for by the individual characteristics of each organ [39]. In the detrusor smooth muscle layer, ICs generate spontaneous excitations independent from the smooth muscle excitation [40]. The fundamental role of the ICs in the bladder lies in the integration of signals between populations of cells, and thus, a relation between changes in ICs and the OAB syndrome is very likely [38]. Bladder overactivity may result from increased excitability in detrusor smooth muscles [41]. The detrusor smooth muscle bundles are surrounded by ICs which integrate signals between different populations of cells and influence the detrusor muscle activity [42]. Hence there may be a relationship between detrusor overactivity and the ICs lying around the muscle bundles. However, overall contractility of detrusor smooth muscles does not only rely on the pacemaking activity of ICs, also nerves may play an important role in the excitability of the muscle cells and the smooth muscles may also have their own excitability mechanism.
In order to further identify the different types of interstitial cells we adopted the classification of Gillespie et al. based on their location [42]. This classification identifies the following subtypes of interstitial cells: Sub-urothelial interstitial cells; located directly below the urothelium [42]. These SU-ICs can be subdivided in Necklace SU-ICs, lying directly below the urothelium, Basal SU-ICs, lying below the necklace SU-ICs and Deep SU-ICs lying below the basal SU-ICs [42]. Between the SU-ICs and the detrusor muscle the Lamina Propria Interstitial Cells are located (LP-ICs) [42]. In the detrusor muscle there are two types of ICs; cells lying around the muscle bundles, the Surface Muscle ICs (SM-ICs) and the cells lying in the muscle bundles, Intermuscular ICs (IM-ICs) [42]. Both types of muscle ICs can be subdivided in inner muscle and outer muscle. At the end of the outer muscle the Muscle Coat ICs (MC-ICs) are located [42].

Unknowns

It has been known for over 30 years that prostaglandins (PGs), produced in both the urothelium and muscle layers, are released from the bladder in response to stretch [43-46]. It has been argued that the PGs play central roles not only in bladder physiology but also in the generation of bladder pathophysiology. This hypothesis is supported by the fact that infusion of PG into the bladder lumen gives rise to an increase in micturition frequency and the incidence of non-voiding phasic contractions between voiding episodes [47-50]. PG production in the bladder serves also other functions. Bachteeva *et al.* reported that PGs play a role in the osmotic water permeability of the frog urinary bladder [51]. Both the details of the mechanisms underlying this action of PGs on voiding frequency and the location(s) where PGs are synthesized in the bladder were unknown until recently. Therefore a study into the localization of cyclo-oxygenase I (COX I), an enzyme which synthesises PGs, was indicated. We found the expression of COX

I predominantly within two general cell systems in the bladder wall: (i) cells within the basal and intermediate layers of the urothelium and (ii) within a population of small cells which are closely associated with a network of vimentin positive cells [4]. These vimentin positive cells presumably interstitial the ICs are present throughout the suburothelial space of the lamina propria and extend over the surface of the muscle bundles which make up the inner layers [4].
The urothelium responds to deformation in a complex manner by releasing not only PGs [47-50], but also adenosine trisphosphate (ATP) [52], nitric oxide (NO) [52-54], and acetylcholine (ACh) [20, 55]. The mechanisms generating these signals and the physiological systems which they target are poorly understood. It has been suggested that the release of these substances is an integral part of a sensory system assessing bladder volume and wall stress [56]. The current concept is that, like PGs, ATP, ACh and NO are released from the urothelium in response to stretch. These substances are then thought to act as transmitters and influence directly or indirectly the suburothelial afferent nerves that are involved in sensation. However, it is possible that there are other targets for these urothelially derived signals.
Autonomous activity in the obstructed guinea pig bladder showed episodes of rapid phasic activity (bursts) [21]. The mechanism involved in controlling the frequency of the motor component of the motor/sensory system, the 'pacemaker', appears to become progressively 'supersensitive' to cholinergic stimulation with the development of pathology [21]. Altered nonmicturition activity might very well contribute to increased afferent output, which in turn contributes to the increased sensations of urge associated with bladder dysfunction.

Aims of the project and long term objective

The concept of the autonomous bladder is well founded and has its basis in a series of publications on the physiological properties and the microstructure of the guinea pig urinary bladder. Fine-tuning this concept to the clinical practice requires that a number of imported questions are resolved. For obvious reasons, many complex measurements and experiments cannot be performed on humans. Similarly, tissue samples from defined areas from the urinary bladder of well characterised patients and normal control subjects are almost impossible to obtain routinely. Therefore, in order to probe basic mechanisms and to study the detailed structure of the urethra and bladder, we are left with the choice of using experimental animals. Nevertheless, using this approach, complex questions concerning the bladder structure and function can be answered when keeping in mind the central thesis in our investigations, i.e. that structure must be related to function.
Previous investigations in our group have clearly shown that in an animal model for the obstructed bladder, overactivity of bladder is accompanied by structural changes in the bladder wall [21]. However, the contribution of each of the changes in the bladder wall that have been observed in relation to the

development of the overactivity remains an enigma. The following questions were the basis for the present investigations:

1) It is known that ICs in the inner and outer muscle respond differently to NO. Are there further subdivisions possible for ICs related to other transmitters that are known to be released in the bladder wall?

2) What changes are there in the properties of the ICs in the obstructed bladder?

3) Is there an influence of the operation on the urethra itself, which is necessary for the obstructed bladder model, on the structure and the functioning of the guinea pig bladder?

4) Where are M_3 receptors in view of the presumed role of these receptors in the overactive bladder syndrome, where are these receptors localized?

Approach

We chose the guinea pig as the experimental animal for our investigations. In previous investigations from our group we used mice, rats, and guinea pigs. However, as a model for the obstructed bladder syndrome, the guinea pig is the animal of choice. There are many similarities between the microstructure of the urinary bladder of the guinea pig and the human [31]. One very important aspect in this respect is the presence of intramural ganglia in the bladder wall of the guinea pig and the human. Intramural ganglia are involved in relaying afferent sensory information [16] and probably also in the pre-processing of this information [16]. We used *in vitro* incubated isolated guinea pig bladder which enabled us to perform pharmacological experiments in combination with immunocytochemistry. The latter technique was our main approach to the localization of nerves, receptors, and cellular components. Although immunocytochemistry can provide very precise information about the localization of specific cellular constituents, i.e. structural proteins or components of signal transduction cascades, it is also a technique which is ill-famous for its artefacts and false positive results. This made it necessary to invest a considerable effort in the characterization of the specificity and applicability of a number of commercially available antibodies. Finally, there was always the self-inflicted requirement of concordance between structure and function.

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Chapter 2

Interstitial cells of the bladder, an overview

S Grol, GA van Koeveringe, PE van Kerrebroeck, J de Vente and JI Gillespie

Introduction

Interstitial cells in the bladder are known for more than 10 years [1]. However the structural components of these cells form a continuing discussion. This review is an attempt to shed some light into this matter. We divided the interstitial cells on their location and identified the structural components of these cells.

Smet *et al.* were the first who described a cell type in the bladder, which responds to stimulation of NO with a rise in cGMP [1]. He named these cells interstitial cells (ICs) [1]. These cells had long dendritic processes extending parallel to the smooth muscle fibres, and contained vimentin, an intermediate filament expressed by cells of mesenchymal origin [1, 2]. The ICs of the bladder have some cytological characteristics in common with the interstitial cells of Cajal (ICC) in the gut. Both ICs and ICC contain bundles of fine cytoplasmic filaments, dense bodies, linear arrays of subsurface vacuoles, and the presence of an interrupted basal lamina [3]. Like ICC, the ICs are vimentin positive and have long dendritic processes [4, 5]. The ICC in the gut play an important role in the control of autonomous activity of the gut. ICC have a distinct morphology; they have long processes and are connected with each other [5].

The gross structure of the bladder wall is well known. On the luminal side we find the urothelium, which is made up of several epithelial cells. The epithelial cells bordering on the luminal site are large flattened cells, and are named umbrella cells. Immediately adjacent to the urothelium lies the lamina propria, which consists of a dense layer of spindle shaped cells also called the suburothelial cell layer. In the part of the lamina propria which borders on the muscle these cells are distributed more sparsely. The lamina propria contacts the inner layer of two layers of criss-crossing muscle layers (figure 1). Although there is a clear difference between muscle cells and the cells of the lamina propria, these layers are intertwined at the bordering zone by the same spindle shaped cells which are often characterized by long processes [6], which appear to be interconnected [7]. These cells have been tentatively identified as myofibroblasts [8], whereas others have termed these cells interstitial cells [1]. Myofibroblasts are characterized by several features; they have a spindle-cell or stellate-cell morphology, a palely eosinophilic cytoplasm, and an abundant pericellular matrix [9]. The immunophenotype of myofibroblasts is: vimentin positive, smooth-muscle actin positive, smooth-muscle myosin negative, non-muscle myosin positive, virtually no desmin and fibronectin positive [9]. Ultrastructurally the myofibroblast has a prominent rough endoplasmic reticulum, a golgi apparatus producing collagen secretion granules, peripheral myofilaments, fibronexus junctions and gap junctions [2, 9].

Morphological features of fibroblasts, myofibroblasts and interstitial cells are more or less the same [10]. Therefore, clear-cut criteria for distinguishing between fibroblasts, myofibroblast and interstitial cells are not available [10]. Komuro suggests that the majority of these cells, if not all, seem to belong to

the same population of cells, which are adapted to special functional needs in their own micro-environment that are peculiar to local, pathological, or experimental conditions [10].
In the literature the terms myofibroblast and interstitial cell have been used interchangeable. In this review we have adopted the name interstitial cell for all cells that are vimentin-positive. We will use the term myofibroblast only when discussing the explicit differences between these two cell types.

Structural immunohistochemical markers used to identify interstitial cells.
Most of the studies aiming at the identification and characterization of interstitial cells have been performed using antibodies against specific epitopes. In general, the specificity of antibodies is not always clear and often difficult to prove. There are many examples of monoclonal antibodies that are specific in Western blotting; however do not work at all in immunohistochemistry. The problems with polyclonal antisera are even bigger: multiple bands will be visible on Western blottings when the protein under study is a constituent in a tissue or cell homogenate. Insufficient blocking of protein degradation will result in epitopes at "incorrect" molecular weights. Unexpected interactions with related or even unrelated proteins have been reported in the literature [11]. Therefore, the question posed almost 25 years ago, is still valid: "Can specificity in immunocytochemistry ever be proven?" [11, 12]. Nevertheless, enzyme- and immunohistochemical methods are the available methods to characterize specific cells in complex tissues. We hold the view that using a number of antibodies in different combinations will result a reliable and reproducible characterisation of interstitial cells in the bladder of a number of species. Antibodies described in the literature to characterize interstitial cells of the bladder will now be discussed. One must realize however that immunohistochemical proof for one or another receptor does not equal function. Given the problem of histochemical specificity, concepts or hypothesis based on histochemical findings will have to be proven with functional experiments. Therefore western blotting or control staining on knock-out animals should be encouraged when immunohistochemical data are presented. In most of the immunohistochemical experiments no antibody controls studies have taken place. We mentioned wether antibody control studies did take place.
The **C-kit** receptor, or also called KIT or CD117, is a cytokine receptor expressed on the surface of haematopoietic stem cells, mast cells, melanocytes in the skin, and interstitial cells of Cajal (ICC) in the gut. This protein is a type 3 transmembrane receptor for mast cell growth factor. C-kit is an important cell surface marker used to identify certain types of haematopoietic progenitors in the bone marrow. Literature on the expression of C-kit on interstitial cells of the bladder is inconsistent. Several authors found C-kit positive ICs in the bladder [2, 6, 13-16], but many other authors couldn't reproduce this. Pezzerone et al. identified C-kit positive interstitial cells in the ureter, but the ICs in the bladder were C-kit negative [17, 18]. This was consistent with the finding of several

other authors who also found C-kit negative ICs in the bladder [18, 19]. These cells were identified with a rise of cGMP after stimulation with NO, or by the use of vimentin [18, 19]. Davidson et al. identified a network of vimentin positive ICs below the urothelium, of which many but not all cells were C-kit positive [19].

Vimentin is a member of the intermediate filament family of proteins, which is a general marker for mesenchymal stem cells. Mesenchymal stem cells are multipotent stem cells that can differentiate into a variety of cell types. One of these cell types are fibroblasts. As described above both fibroblasts and ICs are stellate or spindle-shaped cells and both express vimentin. Thus differentiation between ICs and fibroblasts can be difficult, and caution should be taken interpretating results of vimentin positive cells.

The **CD34** molecule is a cluster of differentiation molecules present on certain cells within the human body. It is a cell surface glycoprotein and functions as a cell-cell adhesion factor. Cells expressing CD34 are found in the umbilical cord and bone marrow as haematopoietic cells, endothelial progenitor cells, endothelial cells of blood vessels, mast cells, a sub-population of dendritic cells. Rasmussen *et al.* describes a subtype of ICs located between the muscle bundles in the bladder that show immunoreactivity for the CD34 molecule [20].

CD44 is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration. It is a receptor for hyaluronic acid. CD44 participates in a wide variety of cellular functions including lymphocyte activation, recirculation and homing, haematopoiesis, and tumor metastasis. Transcripts for this gene undergo complex alternative splicing that results in many functionally distinct isoforms; however, the full-length nature of some of these variants has not been determined. Ozturk *et al.* found that CD44 is expessed on cells in the interstitial space, possibly interstitial cells [21]. Te function of CD44 in these cells is not known.

The number of structural markers to characterize interstitial cells is limited. The results are not unequivocal. Current literature does not provide any data that support the presence of interstitial cells of Cajal in the bladder. We conclude that the ICs in the bladder are best characterized by a combination of the presence of vimentin-immunoreactivity together with the morphological appearance of elongated cells somata showing a variable number of processes with variable length and branching patterns.

Naming of Interstitial cells

The terminology for ICs, proposed by Gillespie et al, based on their location has been used in this review [22]. Figure 2 shows an overview of this classification. ICs directly below the urothelium were named sub-urothelial ICs (SU-ICs), and these could be further subdivided in a) Basal SU-ICs: SU-ICs lying below the necklace cells (the deepest layer of urothelial cells), and b) Deep SU-ICs; SU-ICs lying between the lamina propria and the Basal SU-ICs. ICs located in the lamina propria were named lamina propria ICs (LP-ICs). The ICs in the muscle

layers were divided in cells lying around the muscle bundles: surface muscle ICs (SM-ICs); and cells inside the muscle bundles: the inner muscle ICs (IM-ICs). The ICs lying on the peripheral zone of the bladder muscle were named muscle coat ICs (MC-ICs). Based on this classification we have attempted to give an overview of the literature regarding the specific structural and functional features of these cells.

Suburothelial Interstitial Cells

SU-ICs are described as a distinctive zone directly below the urothelium [23-25]. All layers consist of long spindle-shaped cells that apparently form a network [2, 17, 19, 22, 23, 26, 27]. Depending upon the region, this zone varies from 2 cell layers in the dome up till 5 cell layers in the lateral wall. Ultrastructurally the SU-ICs contain bundles of fine cytoplasmic filaments, dense bodies, linear arrays of subsurface vacuoles, and an interrupted basal lamina.

C-kit-IR on SU-ICs have been reported both positive [15, 16, 19, 26, 28] and negative [19, 28]. Using functional parameters a further subdivision can be made of the SU-ICs. Immediately below the urothelium there is a single layer of interstitial cells that are also immunoreactive for cGK-I [29]. These cells were termed necklace cells. As these cells have extended linking processes this also suggests a network. Although the necklace cells contain cGKI, they did show cGMP-IR in respond to NO [29]. There are several possibilities why no cGMP-immunoreactivity was observed in the necklace cells. First, cGMP levels might be too low, due to high phosphodiesterase (PDE) activity, to be detected by the cGMP-antibody [22]. However, there are no data on PDE activity in these cells. Secondly, although the soluble guanylyl cyclase is present in these cells, it might be in an inhibited form [30].

The cells below the necklace cells stain intensely with cGMP after stimulation with NO [19, 22, 25, 29-32]. The specificity of the cGMP antibody is described in a seperate study [33]. The Deep SU-ICs are located below the layer of Basal SU-ICs. These cells do not show a prominent response to NO with a rise of cGMP, but do contain cGKI. This latter observation suggests that cGMP-signalling plays a role in these cells [29, 30].

It has been demonstrated that the NOS enzyme is located in the SU-ICs [34]. This enzyme can be activated by acetylcholine and ATP. The urothelium releases signalling substances, like acetylcholine [35, 36], ATP [37], and prostaglandins [38-41]. Thus the acetylcholine and ATP derived from the urothelium could activate the SU-ICs.

Both the M_2 and M_3 muscarinic acetylcholine receptors have been reported to be present on SU-ICs [42-45]. Two of these studies were performed by the authors. The specificity of the M_3-antibody used in these studies were established by both western blots and immunohistochemistry of the antibody preincubated with the appropriate blocking peptide. The M_3 immonoreactivity in the sub-urothelial interstitial cells indicates that these cells can be activated

by acetylcholine. However, the role of the muscarinic receptors in the SU-ICs is not clear. Although muscarinic receptors play an important role in the mediation of bladder contractions, it is not apparent that SU-ICs are involved in acetylcholine-controlled muscle contractions. However, SU-ICs also interact with unmyelinated axonal varicosities of cholinergic sensory nerves containing a mixture of clear and large dense-core vesicles, or clear vesicles alone [3, 15, 46]. The identification of these vesicles indicates the release of acetylcholine alone (clear vesicles) or acetylcholine and ATP (dense-core vesicles) [3].

Purinoreceptors have been identified on the SU-ICs [23, 47]. The predominant purinoceptor is P2Y6, and there was weak expression of P2X3, P2Y2 and P2Y4 [23, 47]. Activation of a P2Y receptor by ATP results in an intracellular Ca (2+) transient, which plays a role in the excitability of SU-ICs. This indicates that the purinoreceptors on the SU-ICs play a role in the excitability of the SU-ICs and thus have a possible role in the autonomous activity of the bladder.

Additionally, the SU-ICs showed immunoreactivity for the vanilloid receptor-1 [48]. Vanilloids are thought to block selectively C-fibres by desensitization, decreasing bladder activity, although the relationship between the vanilloid receptors on the SU-ICs and C-fibre desensitization is unclear. Further research is necessary in order to understand the role of the vanilloid receptor on the SU-ICs

Another feature of ICs is that they are inter-connected through connexin-43 containing gap junctions [2, 16, 23, 27, 28, 42]. Sui *et al.* used a well characterised anti-connexin-43 antibody [28]. This antibody was characterised by immunofluorescence, Western blotting of connexin transfectants, and by immunogold electron microscopy [49]. The role of the connexin-43 positive SU-ICs is unknown but their coupling by gap junctions implies that they have the capacity to form a functional syncytium in the suburothelial space. E-cadherins are located on SU-ICs on areas where SU-ICs come in close contact with each other [21, 50]. Cadherin-11 most probably plays an important role in the intercellular physical coupling of SU-ICs [50]. The specificity of the cadherin-11 antibody was established in this by internal and external controls; however a no knockout, blocking peptide or western blot studies were preformed. SU-ICs also stain for CD44; this cell-surface glycoprotein is also involved in cell-cell interactions [21]. CD44 participates in a wide variety of cellular functions including lymphocyte activation, recirculation and homing, haematopoiesis, and tumor metastasis. The function of CD44 on the SU-ICs is not known.

Surface Muscle Interstitial Cells (SM-ICs)

SM-ICs are described as cells located on the edge of smooth muscle bundles, containing lateral branches that run in parallel with the muscle bundles [2, 6, 17, 19, 51, 52]. These lateral branches connect adjacent muscle bundles and also connect to the Muscle Coat Interstitial Cells (MC-ICs) [22]. A close association between enteric nerves and SM-ICs has been described [2, 6, 19].

Ultrastructurally the SM-ICs contain nuclei with clumped peripheral heterochromatin and a dilated rough endoplasmic reticulum with a moderately electron dense amorphous content and prominent Golgi complexes [51].
A sub-population of the SM-ICs were C-kit positive [6, 15-17, 19, 20, 51, 53]. The specificity of the C-Kit antibody has been established using antiserum specificity control experiments [51]. The outer muscle SM-ICs react to NO with a rise of cGMP [18, 22, 31, 54, 55], while this was not the case for the inner muscle SM-ICs. The specificity of the cGMP antibody used was established in a different study [33]. This indicates heterogeneity of these cells. A subset of SM-ICs showed immunoreactivity to the vanilloid receptor 1 and nNOS [34, 48]. Also positive staining for CD34 has been reported [20]. These CD34 positive cells showed no C-Kit immunoreactivity [20].

Intramuscular Interstitial Cells (IM-ICs)
IM-ICs are located throughout the smooth muscle bundles [51]. They have a stellate-like morphology [19, 22, 51] and are in close contact with cholinergic nerve fibres [55]. Ultrastructurally the IM-ICs were comparable with the SM-ICs as they had nuclei with clumped peripheral heterochromatin, a dilated rough endoplasmic reticulum with a moderately electron dense amorphous content, and prominent Golgi complexes [51].
As mentioned before, studies using C-kit as a marker reported contradictory results; C-kit immunoreactivity has been described in the IM-ICs [15, 20, 51, 53], while other authors reported no C-kit immunoreactivity in these cells [18]. One study reported that a subset of IM-ICs stained for C-kit while another subset was C-kit negative [19]. The IM-ICs of the outer muscle also increase synthesis of cGMP when they are stimulated with NO, while the inner muscle IM-ICs do not show a visible response in cGMP after NO stimulation [19, 22, 54, 55]. The specificity of the cGMP antibody was established in a different study [33]. Surprisingly, immunohistochemical staining for choline acetyltransferase (ChAT) showed immunoreactivity in a subset of the IM-ICs [55]. Presently little data is available on other neurotransmitter receptors on this subtype of ICs. Stainings done with an antibody to CD34 showed positive staining in a subset of these cells [20].

Muscle Coat Interstitial Cells (MC-ICs)
MC-ICs are located on the boundary of the outer muscle layer. Little is known in the literature of the MC-ICs. It has been reported that, in a number of species, these cells react to NO with a rise in cGMP [22], but also after incubation of tissue with atrial or brain natriuretic peptide, but not to C-type natriuretic peptide [30]. The specificity of the cGMP antibody was esablished in a different study [33].

Immunohistochemical markers used to characterize interstitial cells.
Cyclic guanosine monophosphate (**cGMP**) is a cyclic nucleotide derived from guanosine triphosphate. cGMP acts is a second messenger molecule. Although cGMP is a small, water-soluble molecule, it can be visualized using immunohistochemistry as highly selective anti-cGMP antisera have been developed against formaldehyde-fixed cGMP (refs). Therefore, these antibodies can be used to locate the target structures for nitric oxide (NO) and natriuretic peptides (refs). There are three known cGMP binding proteins: cGMP dependent protein kinases (CKG), cGMP binding phosphodiesterases (PDEs), and cyclic nucleotide-gated ion channels [56].
cGMP-dependent protein kinases (CGK) are target molecules for cGMP. There are two different CKG genes that produce two isoforms of CGK i.e. **CGK-I** and **CGK-II**. These enzymes phosphorylate a number of biologically important targets and are implicated in the regulation of smooth muscle relaxation, platelet function, sperm metabolism, cell division, and nucleic acid synthesis. Antibodies against CGK-I and CGK-II have been used on bladder tissue (refs). These antibodies have been extensively characterized by biochemical means (refs). One obvious difference in the location of cGK-I and cGK-II is that CGK-II has not been observed in the smooth muscle of blood vessels (refs).
Nitric oxide synthase (**NOS**) is the enzyme that synthesizes NO from the terminal nitrogen atom of L-arginine in the presence of NADPH and O_2. There are three different isoforms of NOS known. NOS-1 (originally called neuronal NOS: nNOS) is constitutively present and activated by calcium-calmodulin. NOS-2 is the inducible form of NOS, also called iNOS. iNOS is expressed in an active form as calmodulin is consitutively bound to the enzyme. iNOS has not been observed in the normal healthy bladder; however, it is expressed at high levels in the obstructed or inflamed bladder. NOS-3, or endothelial NOS (eNOS) is expressed in endothelial cells and needs to be activated by calcium-calmodulin. There are a large number of antibodies available against the three isoforms of NOS. Also, antibodies have been developed which recognize in principle all NOS isoforms, so-called pan-NOS antibodies [34, 57].
Choline acetyltransferase (**ChAT**) is the enzyme that synthesizes acetylcholine. Antibodies to ChAT are well characterized, and performance of these antibodies in the bladder can be checked by performance against cholinergic innervation in the central nervous system (refs). In the bladder ChAT has been described to be present in the cholinergic fibres innervating the bladder muscle and occasional the urothelium, the ganglion cells. In addition, ChAT-immunoreactivity has been observed in umbrella cells lining the lumen of the bladder (refs). Instead of ChAT antibodies one can also use antibodies against the vesicular acetylcholine transporter.
Muscarinic receptors are present in the bladder. Up to date the $\mathbf{M_2}$ and $\mathbf{M_3}$ receptor have been described in this tissue. However, the location of the M_3 receptor is still controversial. This is in part caused by the lack of agreement on the selectivity, or specificity of the antibodies that are currently available. In our

own study have used a range of antibodies against the M_3 receptor from different suppliers, and the results were sometimes widely different [44]. Nevertheless, the abcam Rabbit anti-M_3 and the Santa Cruz Goat anti-M_3 antibodies give a reproducible labelling of the M_3 receptor [44, 45]. Controls of the anti-M_3 antibody used in these studies were performed by western blot and immunohistochemical studies of the antibody with the approprate blocking peptide [44].

P2Y receptors are a family of purinergic, G protein-coupled receptors, which are stimulated by ATP, ADP UTP, UDP and UDP-glucose. P2Y receptors are present in almost all human tissues where they exert various biological functions based on their G-protein coupling.

The **vanilloid** receptor is a type of nerve receptor which is closely related to cannabinoid receptors [58]. Vanilloid receptors are activated by a ligand, which will communicate a sensation within the nervous system. In the bladder the vanilloid receptors subtype 1 (TRPV1) and 2 (TRPV2) is found [16]. This receptor is involved in the transmission and modulation of pain, as well as the integration of diverse painful stimuli [59, 60]. The TRPV receptors in the bladder are thought to be involved in decreasing bladder activity by blocking C-fibres by desensitization.

Immunohistochemistry	SU-ICs	SM-ICs	IM-ICs	MC-ICs
C-kit	some +	some +	Some +	
Vimentin	+	+	+	+
cGKI	+			
cGMP	Necklace: - Basal: + Deep: +	Outer muscle: + Inner muscle: -	Outer muscle: + Inner muscle: -	+
Connexin 43	+	+		
E-cadherin	+			
CD44	+			
Alpha-SMA	+	-	-	
ChAT			Some +	
nNOS	+	some +		
P2X3, P2Y2, P2Y4 P2Y6	weakly +			
M2 receptor	+			
M_3 receptor	+			
Vanilloid receptor-1	+	some +		

Table 1. Immunohistochemical reactivity (IR) on the different types of ICs. Different immunohistochemical markers, seen in the interstitial cells of the bladder, are seen in the left collum. On the top row are the different types of interstitial cells; sub-rothelial interstitial cells (SU-ICs), surface muscle interstitial cells (SM-ICs), intra muscular interstitial cells (IM-ICs) and muscle coat interstitial cells (MC-ICs). + shows that that type of ICs shows immunoreactivity to the marker written on the left. – shows that that type of ICs do not show immunoreactivity to the marker written on the left side. cGMP-IR showed subtypes of interstitial cells; the SU-ICs can be devided into necklace SU-ICs, basal SU-ICs and deep SU-ICs; and the muscle interstitial cells can be divided into outer muscle interstitial cells and inner muscle interstitial cells. When there are no reports of a certain marker on a specific interstitial cell type the box is left blank.

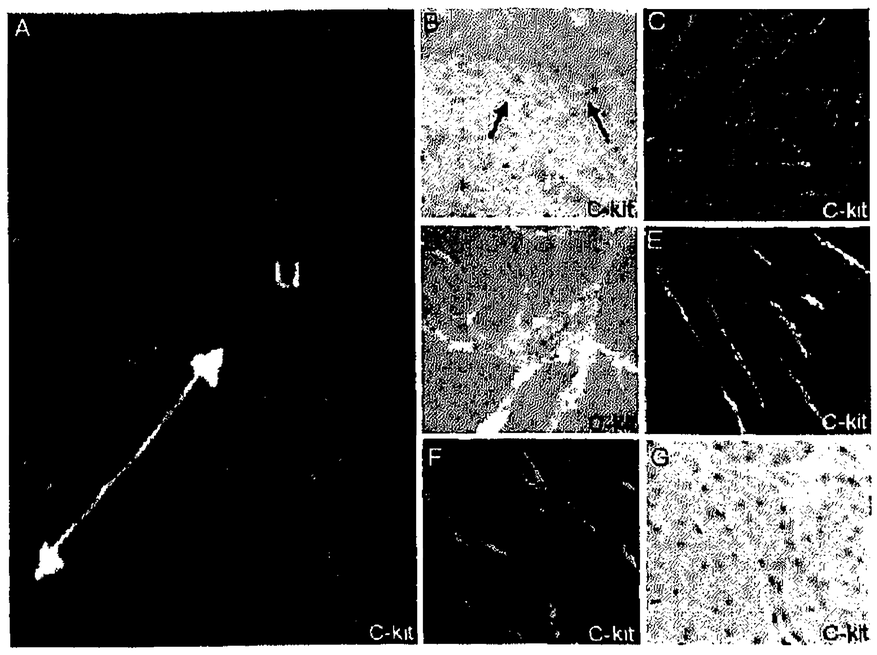

Figure 1. C-Kit immunoreactivity (IR) in interstitial cells (ICs). Panel A shows a picture from a study by Davidson *et al.* [19]. C-Kit IR is visible in sub-urothelial ICs (SU-ICs) and lamina propria ICs (LP-ICs). Panel B shows a picture from a study by Kubota *et al.* [64]. C-kit IR is visible in SU-ICs (arrows). Panel C shows a picture from a study by McCloskey *et al.* [6]. C-kit IR is visible in ICs in the muscle layer. Panel D shows a picture from a study by Biers *et al.* [13]. C-kit IR is visible in ICs in the muscle layer. Panel E shows a picture from a the same study as C [6]. C-kit IR is visible in ICs in the muscle layer. Panel F shows a picture from a study by Davidson *et al.* [19]. C-kit IR is visible in ICs in the muscle layer. Panel G shows a picture from the same study as panel B [64]. C-kit IR is visible in surface muscle ICs (SM-ICs).

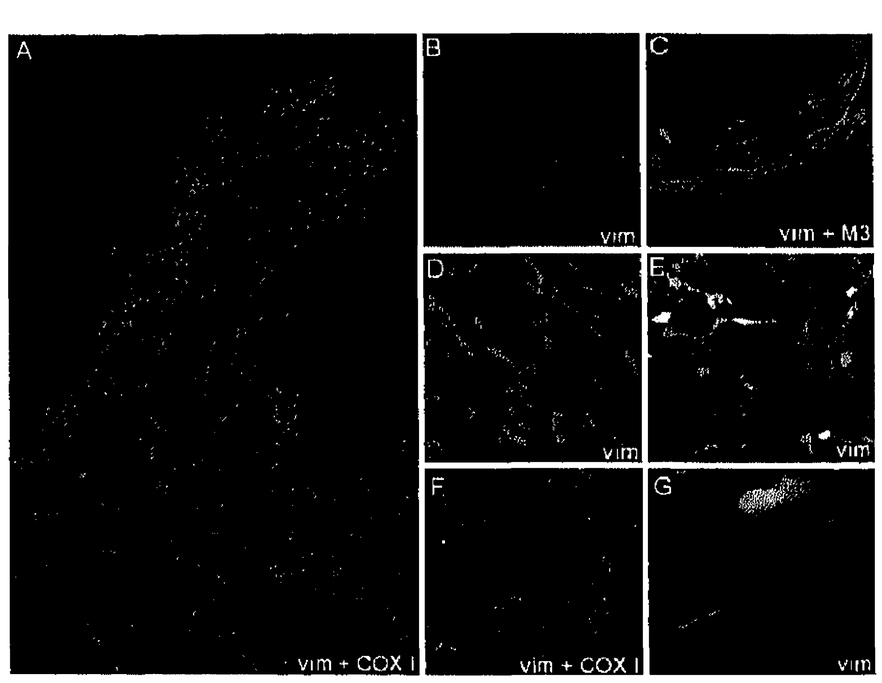

Figure 2. Vimentin immunoreactivity (IR) in interstitial cells (ICs). Panel A shows a picture from a study by de Jongh *et al.* [65]. Vimentin positive sub-urothelial ICs (SU-ICs) are visible directly below the urothelium (green). Below this layer the vimentin positive lamina propria ICs (LP-ICs) are visible (green). COX I IR is visible in red. The basal urothelial layer shows strong COX I IR. A subset of the LP-ICs show IR for COX I. Panel B shows a picture from a study by Sui *et al.* [47]. The SU-ICs and LP-ICs show IR for vimentin (red). Panel C shows a picture from a study by the authors [45]. Vimentin IR in the SU-ICs is visible in green. These cells also show IR for the M_3 receptor (red). Panel D and E shows a picture from a study by Davidson *et al.* [19]. Panel D shows vimentin IR in the LP-ICs (red). Panel E shows vimentin IR in ICs of the muscle (red). A subset of these cells also stains for C-Kit (jellow). Nuclei are visible in purple. Panel F shows a picture from the same study as A [65]. Vimentin IR is visible in surface muscle ICs (SM-ICs, green). A subset of these cells also show IR for COX I (red). G shows a picture from a study by Smet et al. [1]. Vimentin IR is visible in ICs in the muscle bundles.

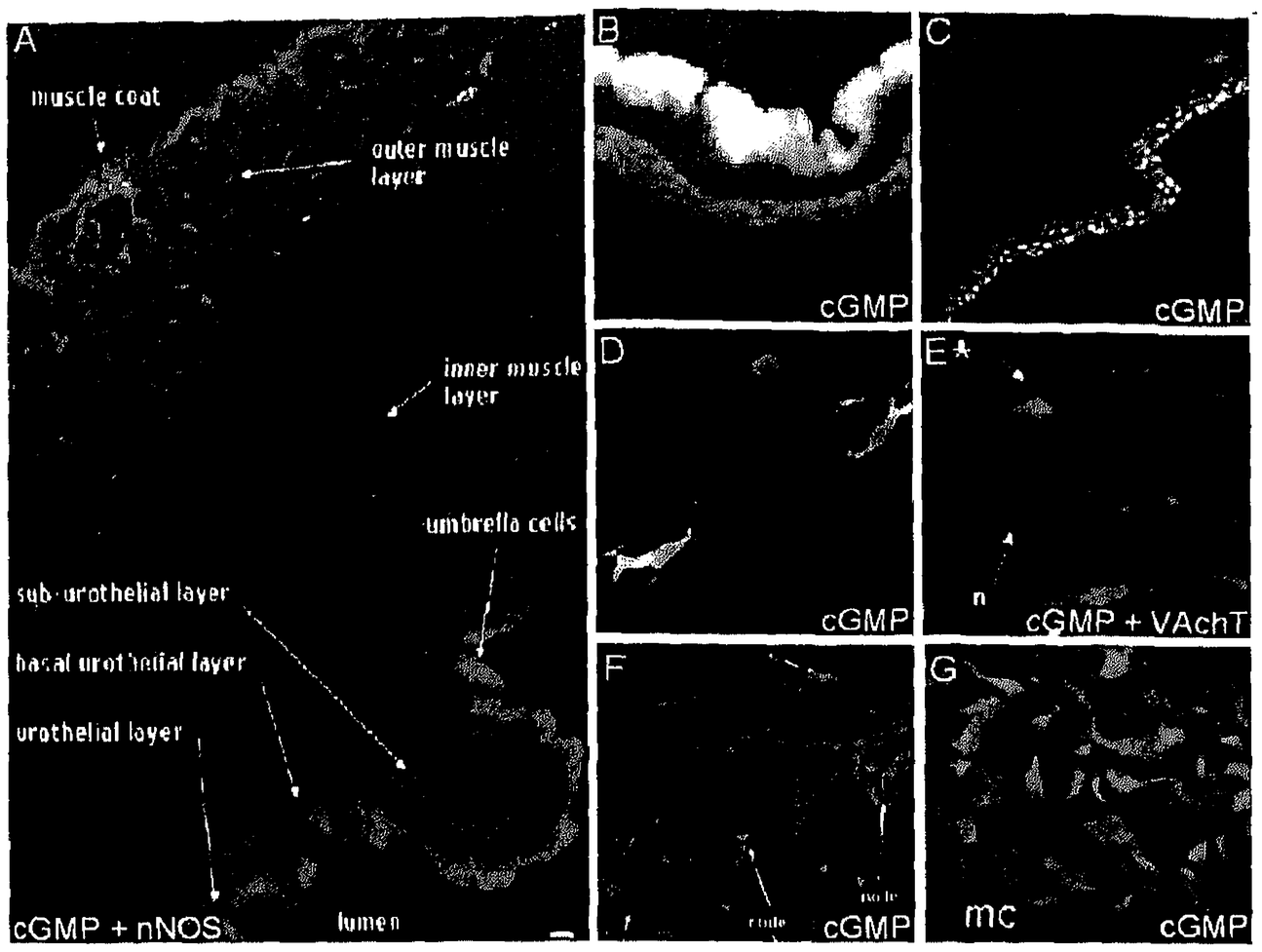

Figure 3. cGMP immunoreactivity (IR) in interstitial cells (ICs) in the bladder. Panel A shows a picture from a study by Gillespie *et al* [22]. cGMP IR is visible in green. ICs of the muscle coat (muscle coat ICs; MC-ICs) and of the outer muscle show cGMP IR, while the ICs of the inner muscle do not show this IR. Both the surface muscle ICs (SM-ICs) and the intra muscular ICs (IM-ICs) of the outer muscle show cGMP IR. The umbrella cells of the urothelium also show cGMP IR. Directly below the urothelium are cGMP positive ICs; the sub-urothelial ICs (SU-ICs). nNOS IR is visible in red. The basal urothelial layer stains intensely for nNOS. A subset of nerves in the lamina propria and muscle layers also show nNOS IR. Panel B shows a picture from a study by Smet *et al* [1]. Panel B shows cGMP IR in the umbrella cells of the urothelium and in cells directly below the urothelium; the SU-ICs. Panel C shows a picture from a study by de Vente *et al.* [30]. cGMP IR is visible in a celllayer directly below the urothelium; the SU-ICs. Panel D shows a picture from the same study as panel B [1]. CGMP IR is visible in ICs in the muscle bundles (IM-ICs) of the outer muscle. Panel E shows a picture from a study by Lagou *et al.* [54]. The same cell types as in panel D are IR for cGMP (star). These cells lie in close relation with VAchT positive nerves (n). Panel F and G show pictures from a study by de Jongh *et al.* [31]. Both panels show pictures from obstructed bladders. Panel F shows cGMP IR in an obstructed bladder. The SM-ICs show IR to cGMP. The number of SM-ICs is increased and these cells are clustered into nodes. Panel G shows a detail of the muscle coat (MC). There is an increase in the number of MC-ICs and all MC-ICs show IR with cGMP.

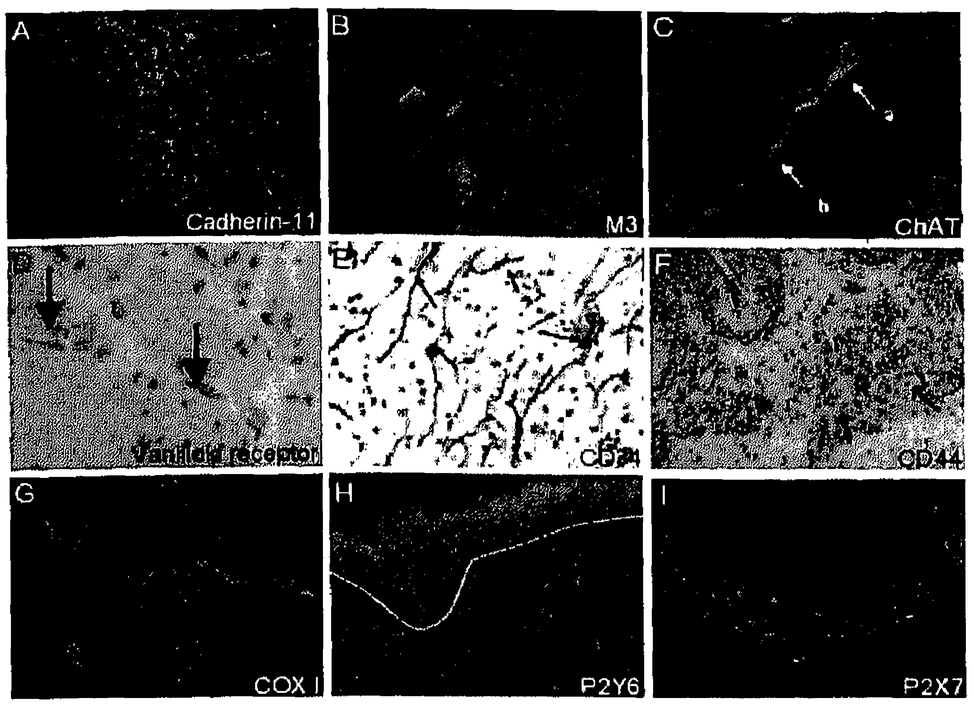

Figure 4. An overview of different antibody stainings on interstitial cells (ICs). Panel A shows a picture from a study by Kuijpers *et al.* [50]. Immunoreactivity (IR) for Cadherin-11 is visible in the ICs. Panel B shows a picture from a study from the authors [45]. IR for the M_3 receptor is visible in red in vimentin positive ICs. Panel C shows a picture from a study by Gillespie *et al.* [55]. IR for choline acetyltransferase is visible in ICs (red, arrow a). These cells lie in close relation with PGP 9.5 IR nerves (green, arrow b). Panel D shows a picture from a study by Ost*et al.* [48]. The vanniloid receptor is visible on ICs (arrows). Panel E shows a picture from a study by Rasmussen *et al.* [20]. The arrows point to CD34 IR ICs around the muscle bundles. Panel F shows a picture from a study by Ozturk *et al.* [21]. The arrows point to an area of interstitial cells that show IR for CD44. Panel G shows a picture from a study by de Jongh *et al.* [32]. COX I IR is visible on ICs. Panel H shows a picture from a study by Sui *et al.* [47]. P2Y6 IR is visible in ICs below the urothelium. The white line seperates the urothelium from the lamina propria. Panel I shows a picture of our own work. P2X7 IR is visible in ICs directly below the urothelium.

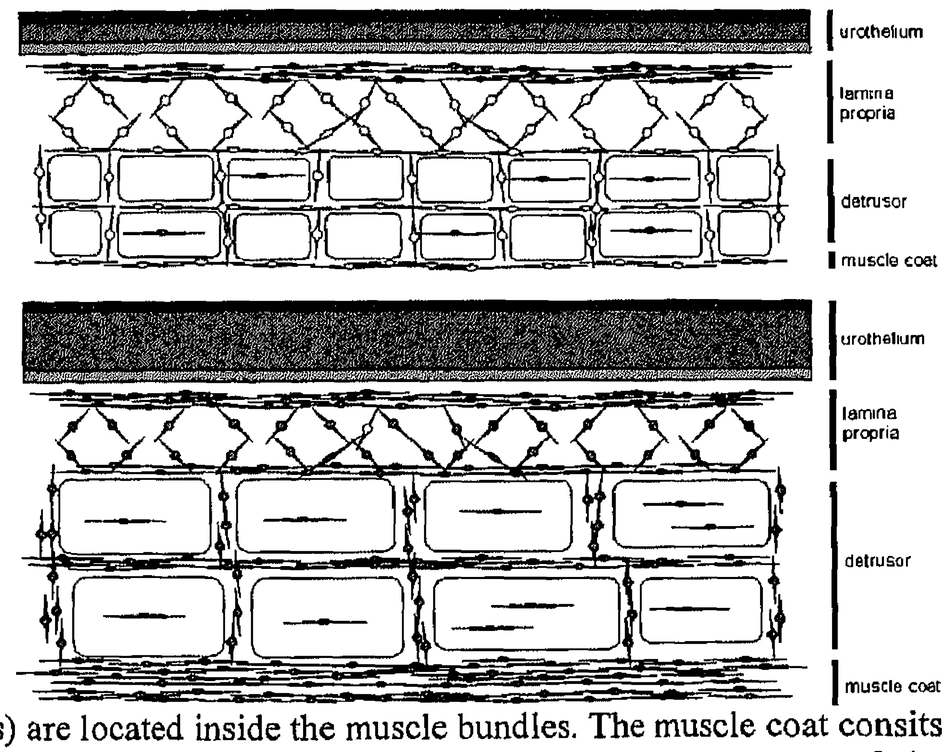

Figure 5. Summarizing a t n f h ges i h bladder wall after bladder outlet obstruction. Panel A is a cartoon of the normal bladder wall. The urothelium consists of 3 layers. Directly below the urothelium the sub-urothelial interstitial cells (SU-ICs) are located. Below these cells lie the lamina propria interstitial cells (LP-ICs). Below the LP-ICs lie the muscle bundles. The surface muscle interstitial cells (SM-ICs) are located around the muscle bundles, while the intra muscular interstitial cells (IM-ICs) are located inside the muscle bundles. The muscle coat consits of a thin layer of muscle coat interstitial cells (MC-ICs). Panel B shows a cartoon of the obstructed bladder. There is an increase in cells in the intermediate urothelium. No difference is noted in the number of SU-ICs, while the SM-ICs and MC-ICs increase dramatically. Furthermore there is a discrete increase in the LP-ICs and IM-ICs.

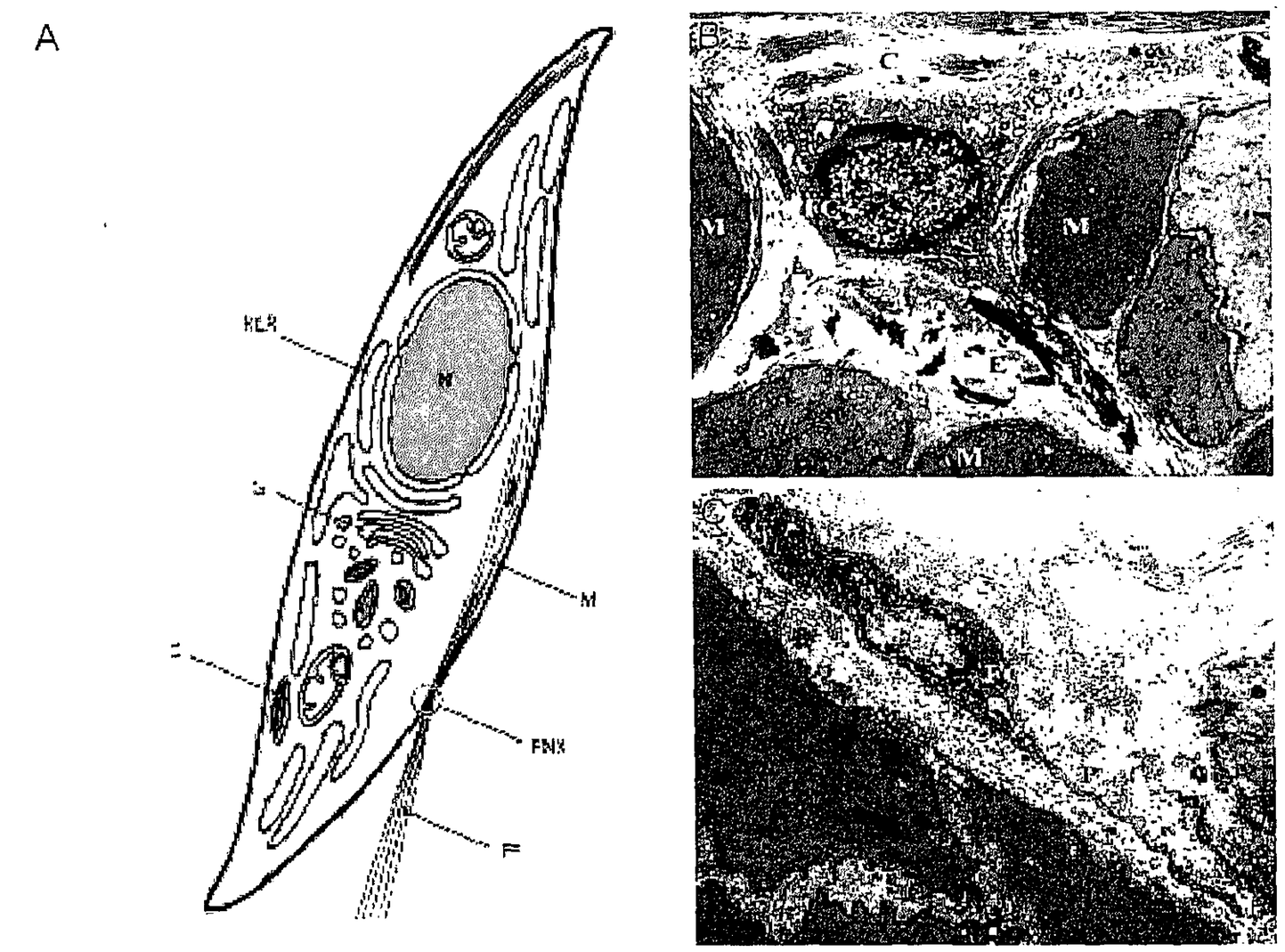

Figure 6. Ultrastructural features of interstitial cells. Panel A shows a cartoon of an interstitial cell (myofibroblast) from a study by Eyden [9]. Abbreviations: AP, attachment plaque; c, collagen secretion granule; FD, focal density; FF, fibronectin fibril; FNX, fibronexus; G, Golgi apparatus; L, lamina; M, myofilament bundle; N, nucleus; RER, rough endoplasmic reticulum; SC, surface (plasmalemmal) caveolae; SPL, subplasma-lemmal linear density (focal adhesion). Panel B and C show electronmicroscope pictures of a study by Drake *et al.*[51]. Panel B shows a multipolar fibroblastic cell at the junction of 3 muscle fascicles (*M*). This cell shows typical cytoplasmic and nuclear appearances with numerous collagen fibrils (*C*) and elastin bodies (*E*). Panel C shows a bipolar fibroblastic cell with flattened process (*P*) extending parallel to adjacent smooth muscle fascicle.

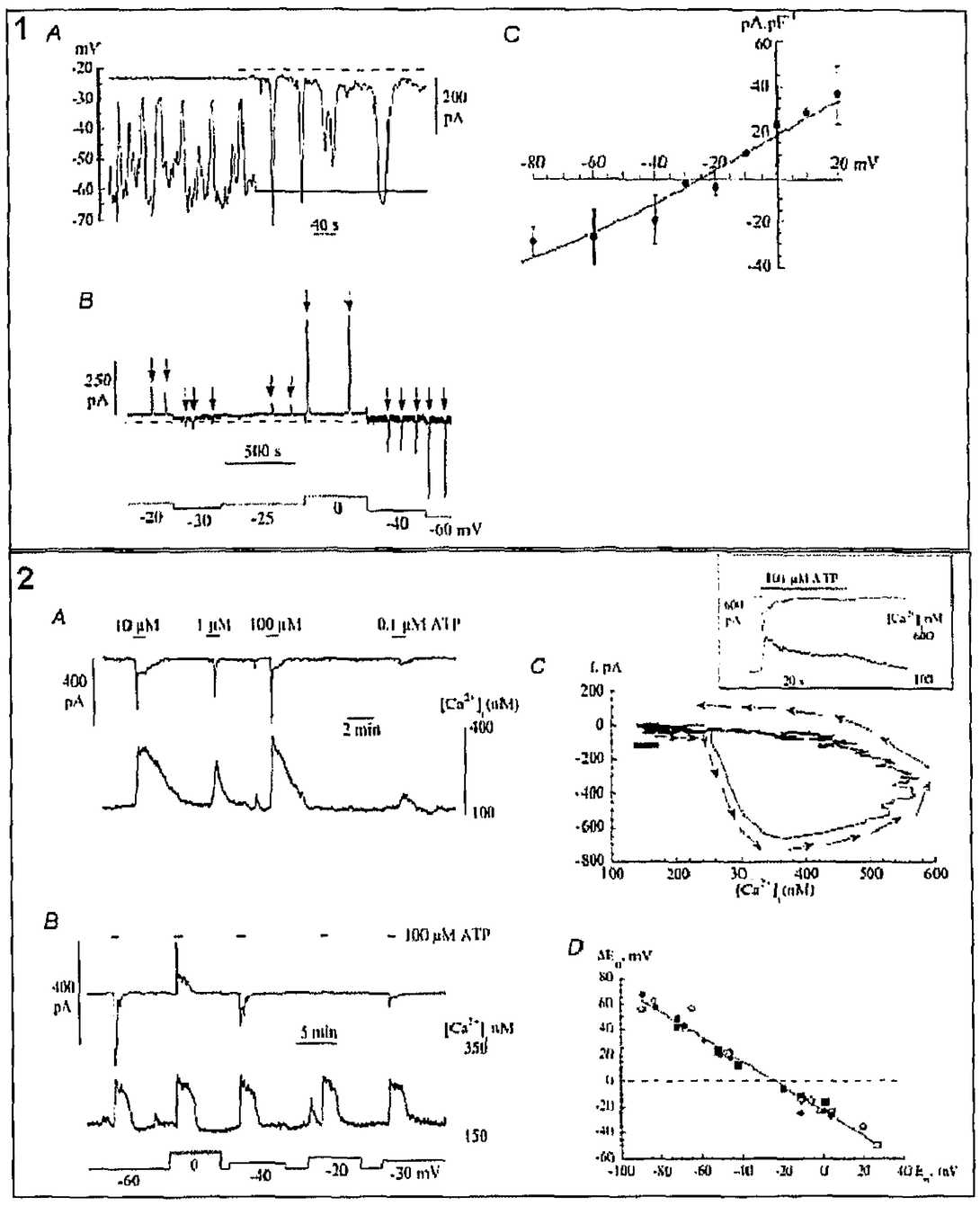

Figure 7. Spontaneous, and ATP-induced Ca2+ transients in suburothelial interstial cells. Panel 1 and 2 show original panels from a study by Wu *et al.* [62]. Panel 1 shows spontaneous currents in suburothelial interstitialcells (SU-ICs). Panel A shows current- and voltage-clamp recordings from an isolated cell using a Cs+-filled pipette. The first half of the record is under current-clamp; the second half shows ionic current recorded when voltage-clamped at –60mV. The dotted line shows the zero-current level. Panel B shows voltage dependence of spontaneous currents under voltage-clamp: holding potential was varied between –60 and 0 mV in this example in a series of steps. Spontaneous currents are arrowed. Panel C shows current-voltage relationship of spontaneous currents from seven cells. Mean ± S.D. of data; the straight line was fitted by least-squares analysis of the mean data.

Panel 2 shows ATP-induced Ca2+ transients and spontaneous currents in SU-ICs. Panel A shows the effect of ATP in concentrations ranging from 0,1 to 100 μM, ATP was added during the periods indicated by the horizontal bars. Cell voltage-clamped at –60 mV with a Cs+-filled pipette. Panel B shows voltage dependence of Ca2+ transients and spontaneous currents induced by 100 μM ATP. Membrane potential was varied between –60 and 0 mV. Panel C shows phase-plane plot of the relationship between current and intrace. The recordings used to generate the plot are shown in the box; arrows indicate the direction of time. The horizontal black bar below the left limb of the plot shows the inherent noise in the traces used to generate the plot. Panel D shows the relationship between the change of membrane potential, Δem, and the initial value, Em, upon addition of 100 μM ATP. The straight line was calculated by least squares analyses. Data are from six separate cells are shown as indicated by different symbols.

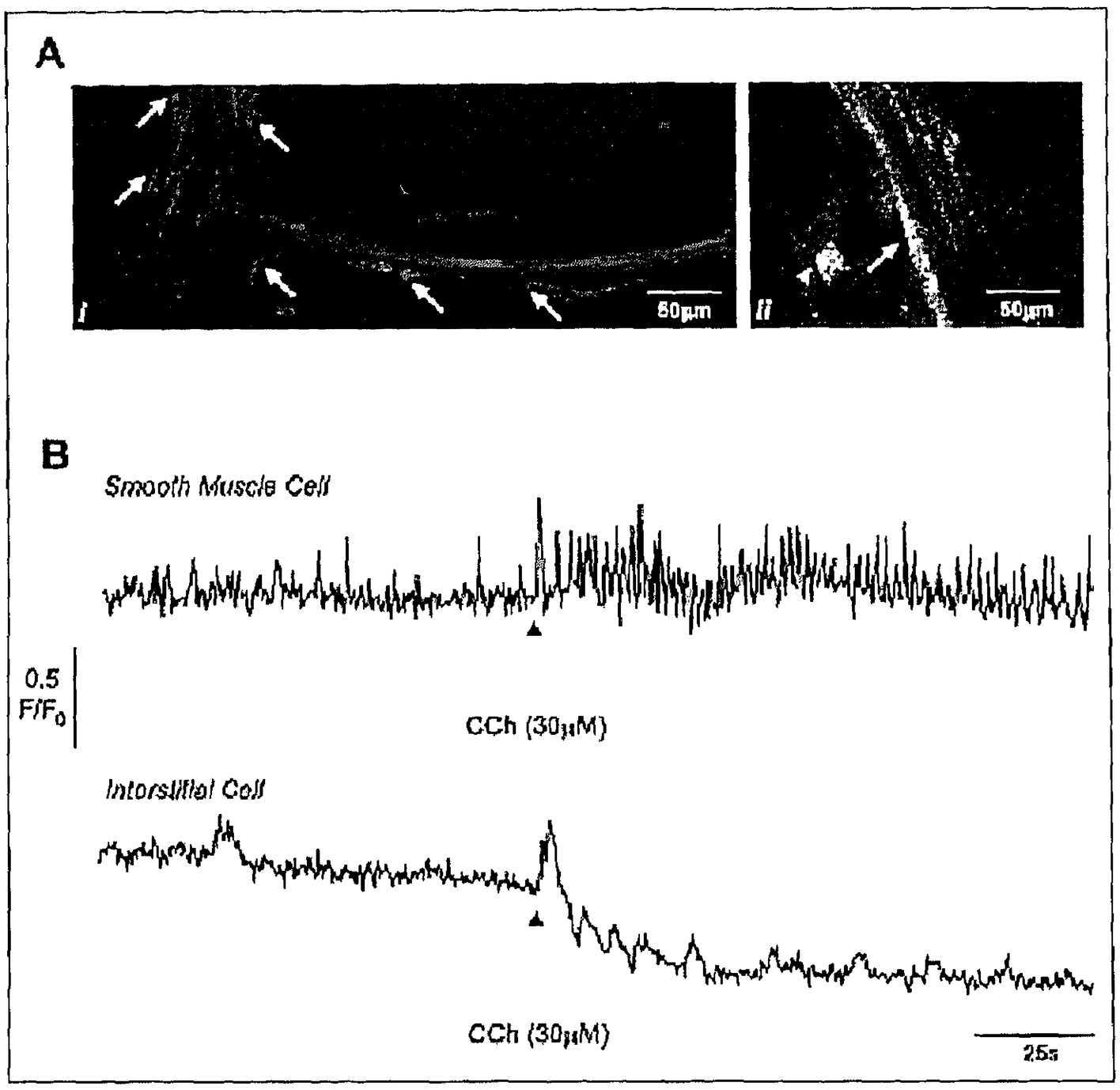

Figure 8. Spontaneous and ATP-induced Ca2+ transients in surface muscle interstitial cells. Panel A and B show original panels from a study by Johnston *et al.* [14]. Panel A*i*. Shows a preparation of a smooth muscle bundle loaded with fluo 4AM (green) and labeled with anti-*c-Kit* (red). The surface muscle interstitial cells (SM-ICs) are visible on the edge of the bundle (arrows). Panel A*ii shows* a SM-ICs (arrowhead) on the left border of a smooth muscle bundle (arrow). Panel B shows the intensity time series from a smooth muscle cell (arrow in *Aii*) and SM-ICs (arrow head in *Aii*) showing the effect of 30 M carbachol. Carbachol increased the frequency of spontaneous transients in the smooth muscle cell and caused an additional increase in intracellular Ca2 in the interstitial cell followed by several oscillations. The reduction in baseline in the interstitial cell record is due to movement of the tissue.

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Chapter 3

Regional differences in sensory innervation and sub-urothelial interstitial cells in the bladder neck and urethra

Simone Grol, Gommert A. van Koeveringe, Jan de Vente, Philip E.V. van Kerrebroeck and James I. Gillespie

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Abstract

Objective The aim of this paper was to identify and characterise possible structural specialisations in the wall of the lower urinary tract in the region of the bladder urethral junction (BUJ) with the specific objective of identifying regional variations in sensory nerve fibres and interstitial cells.

Materials and methods The bladder base and urethra was removed from male guinea pigs killed by cervical dislocation (n=5). Tissue pieces were incubated in Kreb's solution at 36°C, gassed with 95% O_2 and 5% CO_2. Tissues were then fixed in 4% paraformaldehyde and processed for immunohistochemistry. The non-specific marker vimentin and the general neuronal marker PGP 9.5 were used to identify interstitial cells and nerve fibres respectively. Specific antibody binding was visualised using the appropriate secondary antibodies.

Results The wall of the lower urinary tract in the region immediately between the bladder base and the urethra, the BUJ, was found to differ in its cellular composition relative to the adjacent areas. PGP positive (PGP^+) nerve fibres, presumptive afferent fibres, were seen to lie within the urothelium running between the epithelial cells. Two general nerve patterns were seen: branching fibres with no varicosities and complex fibres with varicosities. Fibre collaterals with varicosities were seen to exit the urothelium and occupy the space under the urothelium adjacent to the layer of sub-urothelial interstitial cells. Interstitial cells in the sub-urothelial layer, lamina propria and around the muscle bundles were identified using vimentin (vim^+-ICs). In the base a small number of vim^+ cells were also PGP^+. In the region of the BUJ there was a decrease in the amount of smooth muscle. In this region, below the lamina propria, there was an area densely populated with vim^+/PGP^+ interstitial cells. Nerve fibres were seen to run between the cells in this region.

Conclusion These structural specialisations with in the urothelium and deeper layers of the BUJ suggest that they might be associated with specific functions. The localised highly branched network of the putative afferent nerves suggests the presence of a local axonal reflexes involving possible crosstalk between the urothelium and sub-urothelial layer. The function of the specialised region of interstitial cells is not known and must await further information on the functional properties of this novel cell type. In summary these observations demonstrate further the cellular heterogeneity of the cells in the lower urinary tract and the complexity of the structures. It is one of the major current challenges in functional urology to understand the relationships between these novel structures and overall bladder and urethral function.

Introduction

Damage to the bladder neck is associated with increased voiding frequency, an increase in bladder excitability and major changes to the thickness of the bladder wall and its cellular constituents [1-7] Typically, these changes result from ligatures or restrictions applied to the urethra that are put in place to

reduce urine flow, an approach designed to mimic bladder outflow obstruction caused by conditions such as benign prostatic hyperplasia [1, 3]. However, there are reports demonstrating that similar changes can occur in animals and humans where the bladder neck is damaged but not obstructed [8, 9]. Such observations suggest that obstruction and any resulting increase in voiding pressures cannot be the only factors responsible for triggering the changes. The possibility has therefore been put forward that the overall changes to bladder function and structure result from the damage to specific structures in the region of the bladder neck [8]. One suggestion that was put forward was that, during the surgical procedure, damage to the ganglia or the ganglionic network in the region of the bladder neck and proximal urethra might trigger changes resulting in bladder pathology. However, the possibility has to be considered that damage to other structures might also be involved.

In order to explore these ideas further we must have a detailed overview of the structure of the lower urinary tract in the region of the bladder neck. We divided the region of the bladder neck in 3 areas; the base; the bladder urethral junction (BUJ) and the urethra. The area of the caudal anterior wall was defined as base. The BUJ was defined as the area below the bladder base and above the urethra. In this paper we have focused upon the intramural nerves and interstitial cells. The observations demonstrate regional variations in the wall structure with localised specialisations of innervation and interstitial cell types. These findings are discussed in terms of their possible physiological significance and potential link with the initiation of bladder pathology.

Materials and Methods

The Institutional Animal Care and Use Committee of Maastricht University approved the animal experiments in this study according to EC guidelines. Guinea pigs (5 males, weight range 480-525g) were killed by cervical dislocation. Urinary bladder including the proximal urethra was removed from each animal and placed in ice-cold Kreb's solution: NaCl, 121.1mM; KCl 1.87mM; $CaCl_2$ 1.2mM; $MgSO_4$ 1.15mM; $NaHCO_3$ 25mM; KH_2PO_4 1.17mM; glucose 11.0mM and gassed with 5% CO_2 and 95% O_2 (pH 7.4). Each bladder was divided in two pieces, a ventral piece and a dorsal piece, and maintained in Kreb's solution containing 1mM of the non-specific phosphodiesterase inhibitor isobutyl-methyl-xanthine (IBMX: Sigma-Aldrich) at 36°C for 30 minutes. Incubations were terminated by immersing bladder pieces in ice-cold fixative solution of 4% freshly prepared depolymerised paraformaldehyde for 120 min at 4°C. Then, tissues were fixed overnight at 4 °C in 0.1 M phosphate buffer with 10% sucrose and the next day the tissues were placed in at 0.1 M phosphate buffer with 20% sucrose at 4 °C and then washed overnight at 4 °C in 0.1 M phosphate buffer with 30% sucrose. The tissues were placed in Tissue-Tek O.C.T. compound to form a single block. This was then snap-frozen in isopentane cooled in liquid nitrogen. Cryostat sections (10 μm) were cut, such

that each section was perpendicular to the urothelial surface. Sections were then thawed on to chrome-alum-gelatin-coated slides and stored at -20°C until use.

Immunohistochemistry Sections were dried for 60 min at RT followed by three washes with Tris-buffered saline (TBS; pH 7.6), and thereafter incubated overnight with primary antibodies at 4 °C. The mouse antibody against vimentin (Sigma-Aldrich) was diluted 1:5000. To visualize PGP9.5 we used rabbit anti-PGP9.5 (1: 2000; Abd Serotec); the selectivity and an estimate of the detection limit of these antibodies have been described previously [10-14]. After overnight incubation with the primary antibodies diluted in TBS containing 0.3% (v/v) Triton X-100 (TBS-T), sections were washed in TBS, TBS-T and TBS; each wash step lasted 15 min. Rabbit primary antibody was visualized using Alexa Fluor 488 donkey anti-rabbit IgG (H+L) conjugate (Molecular Probes), diluted 1: 100 in TBS-T. Mouse primary antibody was visualized with Alexa Fluor 594 donkey anti-mouse IgG conjugate (Molecular Probes), diluted 1:100. Sections were incubated with the secondary antibodies for 90 min at RT in the dark. After washing with TBS-T, and TBS, sections were mounted with TBS-glycerol (80%). Typically, for each bladder the staining was done in duplicate and repeated at least 2 separate days. Observations were accumulated from the different slides and from the different bladders.

Sections were analysed and photographed using an Olympus AX70 microscope using a x4, x10, x20 and x40 (oil) objective. For the detection of Alexa 488 fluorescence we used a narrow band-pass MNIBA-filter and for the detection of Alexa 594 we used a filter with a narrow excitation band, the U-M41007A filter (both filters are from Chroma Technologies). The microscope was equipped with a cooled CCD Olympus Digital video camera F-view. Images were stored digitally as 16 bits images by using the computer program cell-P® (Olympus, Germany). The number of grey values was reduced using a linear function into 4095. Images were arranged with the program Adobe Photoshop 5.5 or 7.0.1 (San Jose, CA, USA).

Results

General observations Figure 1 clarifies the regions investigated in this study and the major findings. Panels A, B and C show low power images of three different bladders of the region between the bladder base and the urethra: the bladder urethral junction (BUJ). Six regions have been identified (1-6): 1) urothelium of the BJU; 2) urothelium of the urethra; 3) suburothelial layer; 4) lamina propria; 5) urethral musculature; 6) BUJ transitional zone. The structural features of these regions will now be described in detail. These observations have been made in all sections of the bladders from 5 normal healthy guinea pigs (see Method sections).

The differences between the urothelium of the BUJ and the urethra are illustrated in panels 1 and 2 of Fig. 1. A striking difference was observed in the innervation pattern of the urothelium: the BUJ urothelial layer is rather well innervated whereas no innervation was observed in the adjacent urothelium of

the urethra. In region 3, immediately below the urothelium, we observed vimentin positive (Vim^+) interstitial cells: the sub-urothelial interstitial cells (SU-ICs; Fig. 1, panel 3). This layer is present in the BUJ as well in the urtheral region and in the bladder base. In region 4, the lamina propria of both regions we observed also Vim^+ interstitial cells in addition to nerve fibres. Strikingly, we did not observed smooth muscle fibres in the BUJ, as opposed to the urethra (region 5). This is illustrated in panel 5 (Fig. 1). These muscle bundles are surrounded by Vim^+ interstitial cells and have a clear innervation. Some of these Vim^+ interstitial cells demonstrate colocalization with PGP9.5. A region which has not been described before is the transition zone between the bladder base and the urethra (Fig. 1, region 6): a region of the wall which is devoid of smooth muscle cells but which contains, in the region below the lamina propria, a dense area of Vim^+ and PGP positive (PGP^+) cells. These cells have the appearance of interstitial cells: nuclei are clearly visible and often several process can be observed. Based on this general structure and their location it is likely that these cells are not neurones and may therefore represent a type of interstitial cell that has not been described before (Vim^+/PGP^+) interstitial cells.

Complex patterns of nerves running within the urothelial layer In the region between the bladder base and the urethra we observed that there was an unusual pattern of nerves running within the epithelial layer of the urothelium. Figure 2 A, B and C show respectively the urothelium of the bladder base, BUJ and urethra. The urothelium of the bladder base is sparsely innervated. Fibres, when present, were occasionally found within the urothelium but more often lying immediately below the epithelial layer (arrows Figure 2 A). There was a large number of fibres running between the epithelial cells of the urothelium of the BUJ (Fig. 2B). In complete contrast, no nerve fibres could be observed in the urothelium of the proximal urethra (Fig. 2C). Panels D, E and F show another three examples originating from different guinea pigs, of the dense innervation of the urothelium of the BUJ. In each panel fibres can be seen to exit the urothelium and enter the sub-urothelial layer. Panel E illustrates a particular feature where the fibres leaving the urothelium are shown to branch, often more than once, with each collateral having distinct varicosities. Fibres with varicosities are also observed within the urothelial layer.

Interstitial cells within the lamina propria and muscle layers Panel A in Figure 3 illustrates a low power image of the wall in the region of the proximal urethra. The region of the lamina propria and muscle layers are easily identified. Panels B and C show that the interstitial cells in the lamina propria are Vim^+. PGP^+ nerve fibres are seen to run between these interstitial cells. Panel C shows the presence of Vim^+ interstitial cell on the surface of the muscle bundles and the presence of PGP^+ nerve fibres within the bundles running between the fibres. Some of the Vim^+ interstitial cells on the surface of the muscle bundles also stain for PGP9.5. Figure 4 picks up a further feature illustrating the Vim^+ interstitial cells of the sub-urothelial layer (sub-urothelial interstitial cells (SU-ICs). In the BUJ and urethra these cells form a layer 2-3 cells deep. These cells

are Vim^+. In contrast, in the bladder base, Vim^+ interstitial cells can be found which are also positive for PGP. This observation raises the possibility that the interstitial cells in this layer are heterogeneous with different types in the different region of the lower urinary tract.

Specialisation of interstitial cells in the BUJ Figure 5 illustrates an unusual and distinctive feature of the wall in the region of the BUJ. As noted in Figure 1 there are few muscle bundles lying immediately below the lamina propria. Instead, this region is occupied with a dense population of spindle shaped cells many cell layers thick. These cells have predominantly oval cell bodies and extend 2 or 3 short processes. All of the cells in this region are Vim^+ and PGP^+. These cells do not have the characteristics of neurones. They appear to be a type of interstitial cell with an unusual immuno-profile. Such cells have not been described previously in the bladder or urethra.

The features highlighted in the images above are brought together in Figure 6 which shows, in cartoon form, the structures of the bladder base, bladder urethral junction and the urethra. The nature and possible physiological significance of these structures are discussed below.

Discussion

This paper describes two new general observations relating to the structure of the wall of the lower urinary tract in the region of the bladder base and urethra: the bladder urethral junction (BUJ). The findings point to specialisations in the structure and distribution of nerve fibres associated with the urothelium and interstitial cells deep in the wall.

Interest in the structures of this region is increasing following the realisation that damage might contribute to changes resulting in general pathology, specifically overactive bladder [4, 6-8] Several structures are obvious candidates for such damage (nerve trunks, superficial and intra-mural ganglia and blood vessels). The present observations add to these possibilities identifying regions of sensory nerve and interstitial cell specialisations that may also contribute.

It is well known that there are regional variations in the innervation of the LUT, particularly the afferent innervation, with a relatively sparse innervation of the dome and lateral wall and a denser innervation of the bladder base [15, 16]. The observations in this paper on the guinea pig bladder are in broad agreement with this concept. In the lateral wall of the guinea pig bladder it has been shown that nerves, presumably sensory nerves are found in the space immediately below the urothelium between the layers of sub-urothelial interstitial cells [17, 18]. Two different types of fibres were identified based on the expression of the enzyme responsible for synthesising acetylcholine (choline acetyl transferase (ChAT)) and calcetonin gene related peptide (CGRP). The majority of these fibres did not penetrate the urothelium but ran parallel to the basal membrane in the space between the urothelium and the sub-urothelial interstitial cells. ChAT and CGRP fibres were also found in this region of the bladder to make contact

with intramural ganglia suggesting the presence of local neural networks involving excitatory and inhibitory inputs [18, 19]. The pattern of nerve fibres found in this study in the bladder base is similar to that described above. However, in this study no attempt was made to identify different types of fibres. A clear difference was observed in the pattern of nerves found in the region lying between the base and the urethra. We have described this region as the bladder urethral junction (BUJ). Here there is a dense population of nerves which lie within the urothelium and which appears to send projections into the sub-urothelial layer. It would appear that there might be two distinct patterns of fibre: fibres with no varicosities and highly branched fibres with varicosities. This difference may reflect different fibre types but this differentiation awaits further study. It is not known whether these fibres are afferent or efferent. Given their location on the urothelium, a structure which is becoming more and more associated with sensory roles [20-22] it is possible they are afferent. However, the possibility cannot be excluded that some fibres may sub-serve an efferent role in modulating urothelial function in this region of the lower urinary tract. Some fibres are seen to exit the urothelium and branch in the sub-urothelial space. The functional significance of this specialisation is not obvious. It may be related to increasing the sensitivity of these fibres to a particular mode of stimulation (e.g. mechanical deformation). Alternatively it might represent regions where collateral fibres of the sensory nerves make contact with specific structures. Such a local reflex involving collaterals of sensory nerves is well recognised in the skin [23] and has been suggested to occur in the lateral wall of the bladder [18, 19]. Irrespective of the precise details of the function of these nerves fibres the fact that there is a clear specialisation in structure and distribution strongly suggests a high degree of specialisation in the function, possibly, sensory, in this region.

In relation to this it is worth speculating about the origin of sensation in the bladder. It has been pointed out that sensations of bladder volume appear to be of different types [24]. In the early stages of bladder filling general sensations are felt in the general region of the bladder and lower abdomen. However, as the volume increases the sensations become more intense, sometimes described as urge sensations, and these now appear to come from lower down in the LUT in the region of the bladder neck and urethra [25, 26]. The growing number of observations suggestion regional differences in the sensory innervation of the bladder wall may provide a structural correlation for these different sensations. Furthermore, it has been demonstrated by Barrington some 80 years ago that activation of sensory fibres from different regions of the LUT contributed to different reflexes (the 7 Barrington's reflexes, [27, 28]. The finding of different types of nerve fibre in the same region and different patterns of innervation in different regions might also provide structural correlates with Barrington's functional data.

There is now a growing interest in structure, distribution and classification of cells in the lower urinary tract known collectively as interstitial cells. Interstitial

cells have been described in the ureter, bladder and urethra [29-33]. These cells are not a homogeneous population and there appear to be distinct populations in the different regions of the LUT. Indeed, even within the same region, there may be several distinct populations of interstitial cells associated with specific structures in the wall [5, 19, 34-36]. At present there is no single marker to identify interstitial cells. The majority can be identified by the expression of Vimentin [5, 34, 37], some express the receptor for stem cell factor (cKit) [38], others are responsive to nitric oxide showing an elevation in cGMP [19, 35] and others express specific patterns of receptors (e.g. the muscarinic receptor M_3 (unpublished data) [39], cyclo-oxygenase (COX I) [6] and prostaglandin receptors (S. Rahnama'i personal communication) [40] These cells are found in the normal bladder but it has been reported that there are major changes in the type and distribution of interstitial cells associated with bladder pathology. This suggests that they may play key roles in bladder physiology and pathology. In the mouse, it has been suggested that one population of interstitial cells may be associated with the modulation of motor activity in the bladder wall [30]. Here, the only cells in the bladder wall which respond to exogenous nitric oxide with a rise in cGMP is a population of interstitial cells associated with the outer muscle layers. Since contractile activity in the whole isolated bladder is reduced by nitric oxide the conclusion has been drawn that these cells are involved in regulating activity in the smooth muscle.

In the guinea pig bladder several distinct populations of interstitial cells have been described [6, 31, 34, 35]. These include a dense layer lying immediately below the urothelium (sub-urothelial interstitial cells (SU-ICs), cells in the lamina propria (lamina propria interstitial cells (LP-ICs), cells surrounding the muscle bundles (superficial muscle interstitial cells (SM-ICs) and cells within the muscle bundles (intramuscular interstitial cells (IN-ICs) [41]. Subsequent studies have shown that even within these broad populations of interstitial cell there may even be sub-populations with different characteristics. For example, the SM-ICs associated with the inner muscle layers express vimentin but do not respond to exogenous nitric oxide with a rise in cGMP. In contrast the SM-ICs associated with the outer muscle layer are vimentin positive but respond to nitric oxide with a rise in cGMP (R de Jongh personal communication) [19, 42]. Similarly, the SU-ICs are distinct from the LP-ICs in that the LP-ICs are associated with the expression of cyclo-oxygenase type I (COX I) while the SU-ICs are not [6].

The present study adds to the complexity of the sub-types of interstitial cells found in the LUT: the description of a population of interstitial cells which are vimentin positive and which express PGP9.5 represents a new type. PGP9.5 is considered to be a non-specific but good marker for nerves. However there are reports that the epitope detected by PGP9.5 is also expressed on mesenchymal cells in the developing embryo [43, 44]. These mesenchymal cells represent a specific population of cells with a distinct function. The present observation that similar cells are found occasionally in the sub-urothelial layer of the bladder

base but predominantly in the interstitium of the BUJ, suggests a particular functional specialisation of these cells in this region. The nature of this function is not known. It may relate to the regulation of extra-cellular matrix or it may have more dynamic functional roles. Interstitial cells have been linked to possible sensory functions [45] while other interstitial cells are excitable and generate electrical activity and to communicate via gap junctions with the smooth muscle to influence contraction. Also some interstitial cells are thought to generate pacemaker activity and influence phasic activity in the bladder [6, 41, 46]. It is possible that the specialised region of interstitial cells in the region of the BUJ might have sensory or pacemaker functions. In this region there is also a reduction in the amount of muscle in the wall. This is a surprising observation. It implies a specialisation but this cannot be associated with any sphincter function of the proximal urethra. One possibility is that this region is less rigid that adjacent regions with muscle and so more able amenable to distension. In conjunction with a possible increase in sensory innervation in this region it may be specialised to respond to distension with the generation of localised afferent nerve discharge. This could contribute to specific and localised sensation as described by Nathan [24] and above.

The present observations add to the complexities of the lower urinary tract. The observation of such localised structural specialisations in nerve and interstitial cells must reflect specific functional specialisation. At present we can only speculate on the role of these structures. However, if we are to understand the detailed physiology of the LUT and the origins, nature and consequences of changes that give rise to bladder pathology we must identify and understand these complexities. This is the next series of challenges in functional urology.

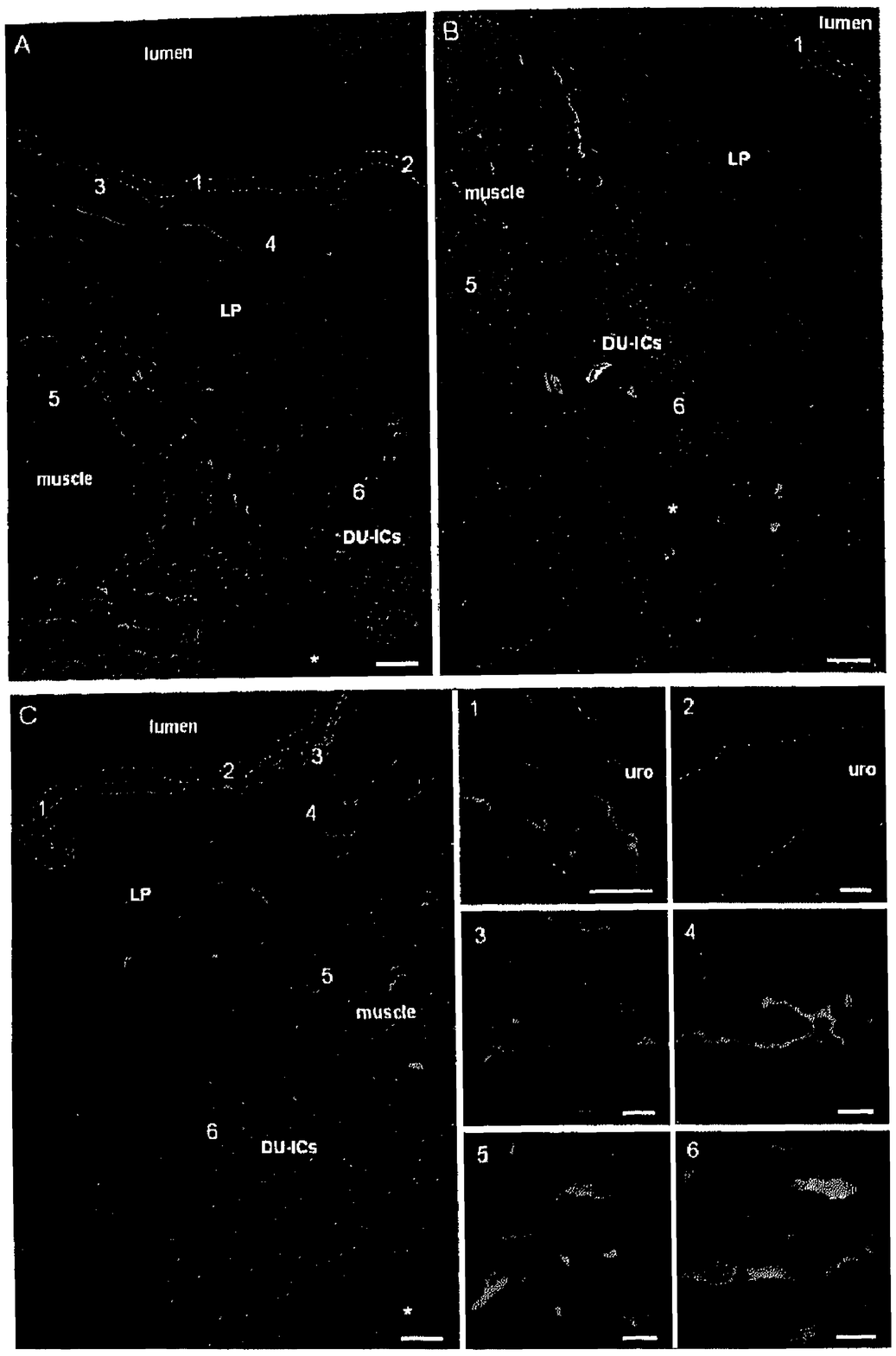

Figure 1. The structural components of the vessel wall in the region of the bladder urethral junction (BUJ). A, B and C show a low magnification picture of the BUJ of three different bladders stained with antibodies for the nerve marker PGP9.5 (green) and vimentin (red), a marker for interstitial cells (ICs). The BUJ is on the left and the urethra on the right. The numbers in the small panels correspond to the location of the panels on the right of panel C. The dotted lines show the location of the urothelium. The star shows the location of the prostatic duct. Panel 1 shows PGP9.5 positive nerves in the urothelium of the BUJ, while the urothelium of the urethra is shown in panel 2. No PGP9.5 positive nerves are identified in this region. Panel 3 shows the vimentin positive ICs just below the urothelium; the sub-urothelium interstitial cells (SU-ICs). Panel 4 identifies the ICs in the lamina propria (LP-ICs). Note the close relation of these ICs with nerves. Panel 5 shows ICs between the muscle bundles of the urethra; surface muscle ICs (SM-ICs). Note that some of these SM-ICs are PGP9.5 positive. Panel 6 shows an area in the BUJ containing a large amount of ICs (deep urothelial interstitial cells – DU-ICs). All these ICs are vimentin positive and PGP9.5 positive. Note the close relation between nerve fibres and the ICs. Calibration bars 100μm in A, B and C and 10 μm in 1,2,3,4,5 and 6.

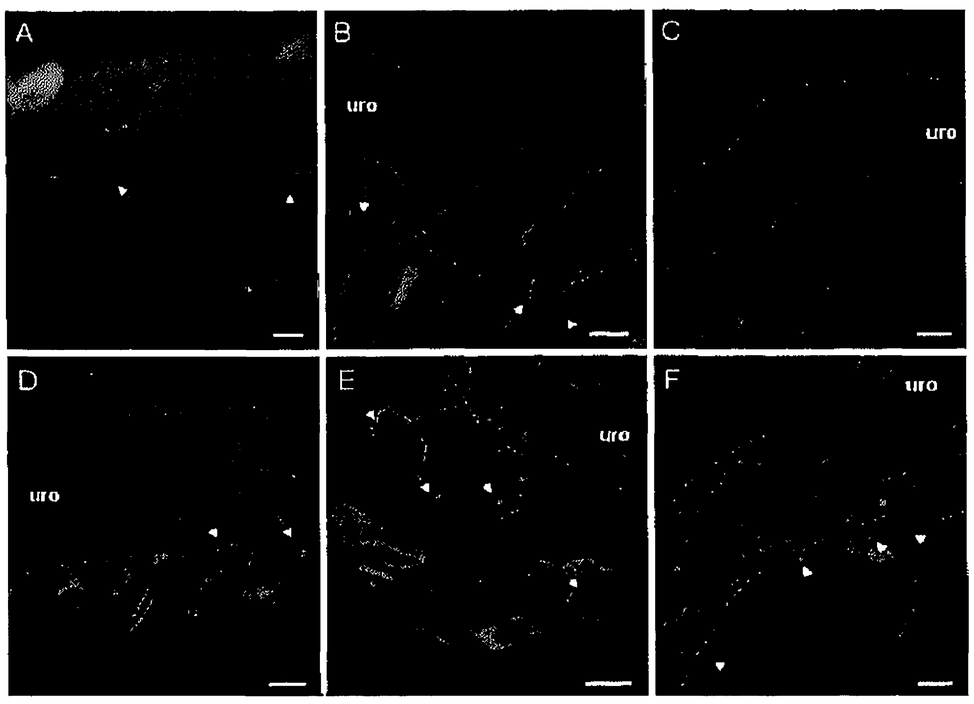

Figure 2. Differences in the distribution of nerves within the urothelium in the region of the bladder urethral junction (BUJ). All panels are stained with the nerve marker PGP9.5 (green) and the ICs marker vimentin (red). Panel A shows the urothelium and sub-urothelium of the bladder neck. Sparse nerve innervation of the urothelium can be identified (arrows). Directly below the urothelium there is a layer of vimentin positive ICs. Note the PGP9.5 positive staining in the urothelium. Panel B shows the urothelium of the BUJ. There is a dense nerve innervation of the urothelium in this area. The arrow points to an urothelial nerve penetrating the sub urothelium. In this area there is no PGP9.5 staining of the urothelium. Panel C shows the urothelium and sub-urothelial layer of the urethra. No nerve innervation of the urothelium can be identified and only sparse nerve innervation of the sub-urothelial layer can be identified. Panel D, E and F show other examples of panel B; the urothelium of the BUJ. The arrows in panel D show urothelial nerves penetrating the sub-urothelium. Note the close relation of these nerves with the SU-ICs. The arrows in E also show urothelial nerves penetrating the urothelium and lying in close contact with SU-ICs. Note the varicosities on these nerves. Panel F shows varicosities of the urothelial nerves in the urothelium. The arrows point to urothelial nerves penetrating the sub-urothelium. Calibration bars 10μm in A, B, C, D, E and F.

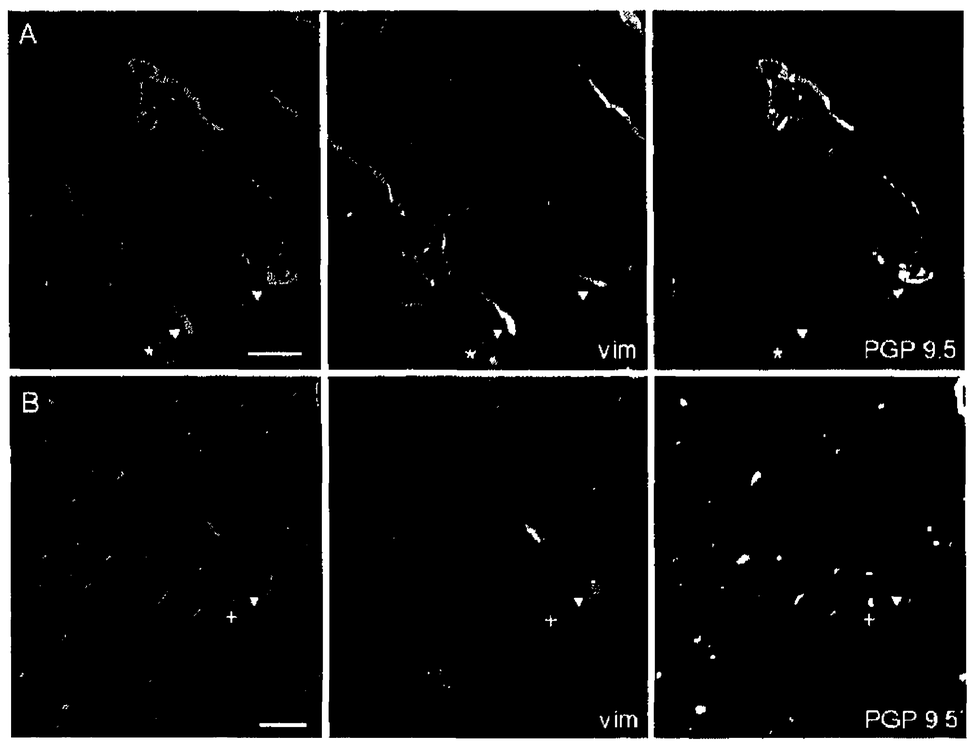

Figure 3. Vimentin positive interstitial cells (ICs) in the lamina propria and muscle in the region of the urethra. Panel A shows an example of the lamina propria in the urethra. The area is stained with the nerve marker PGP9.5 (green) and vimentin a marker for ICs (red). PGP 9.5 positive nerves in close contact with vimentin positive ICs (arrow). Note the absence of co-localisation between vimentin and PGP9.5 in the ICs (*). Panel B shows an example of the muscle in the urethra. Small nerves can be seen in the muscle bundles. Around the muscle bundles vimentin positive ICs can be seen; surface muscle interstitial cells(SM-ICs). Note that some of these SM-ICs stain for both vimentin and PGP9.5 (+). Calibration bars 20 μm in A and B.

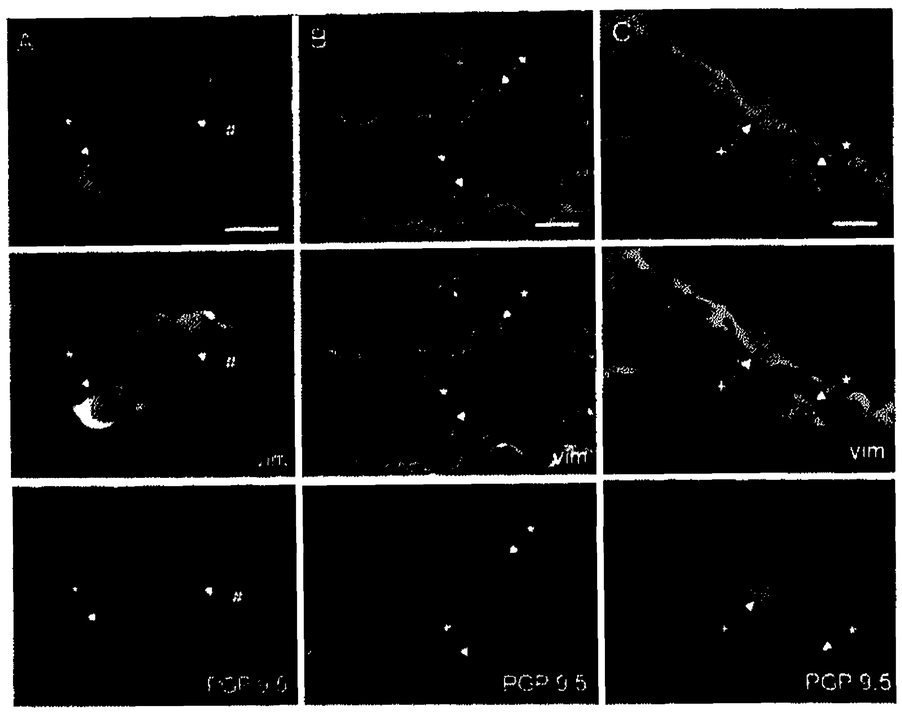

Figure 4. Vimentin positive cells in the sub-urothelial layer in the region of the bladder urethral junction (BUJ), the urethra and the bladder base. All panels are stained with the nerve marker PGP9.5 (green) and vimentin a marker for ICs. Panel A shows an example of the sub-urothelial layer of the BUJ. Vimentin positive sub-urothelial interstitial cells (SU-ICs, *) and PGP9.5 positive nerves (#) can be seen. Note the absence of PGP9.5 staining in the SU-ICs. Panel B shows an example of the sub-urothelial layer of the urethra. Vimentin positive SU-ICs can be seen (*). No co-localization between vimentin and PGP9.5 is identified in this area. Note the sparse innervation of PGP9.5 positive nerves. Panel C shows the sub-urothelial layer of the bladder base. In this area two types of SU-ICs can be identified; vimentin positive, PGP9.5 negative SU-ICs (*) and vimentin positive, PGP9.5 positive SU-ICs (+). Calibration bars 10μm in A, B and C.

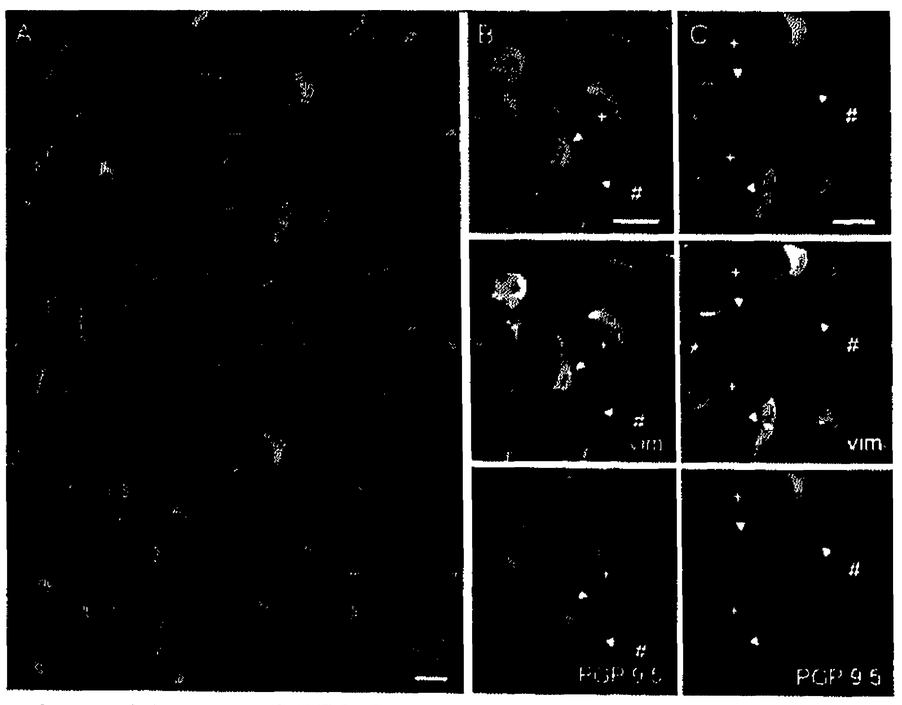

Figure 5. Vimentin positive and PGP9.5 positive cells in the wall of the bladder urethral junction (BUJ). Panels A, B and C are stained with the nerve marker PGP9.5 (green) and vimentin (red), a marker for interstitial cells (ICs). A shows the area below the lamina propria in the BUJ (region 6 in Fig. 1). In this area lots of ICs can be found. All these ICs are vimentin positive and PGP9.5 positive. The boxes in panel A are shown in larger magnification in panel B and C. Note the co-localisation of vimentin and PGP9.5 in the ICs of this region and note the close relation of PGP9.5 positive nerves with these ICs. Calibration bars 10μm in A, B and C.

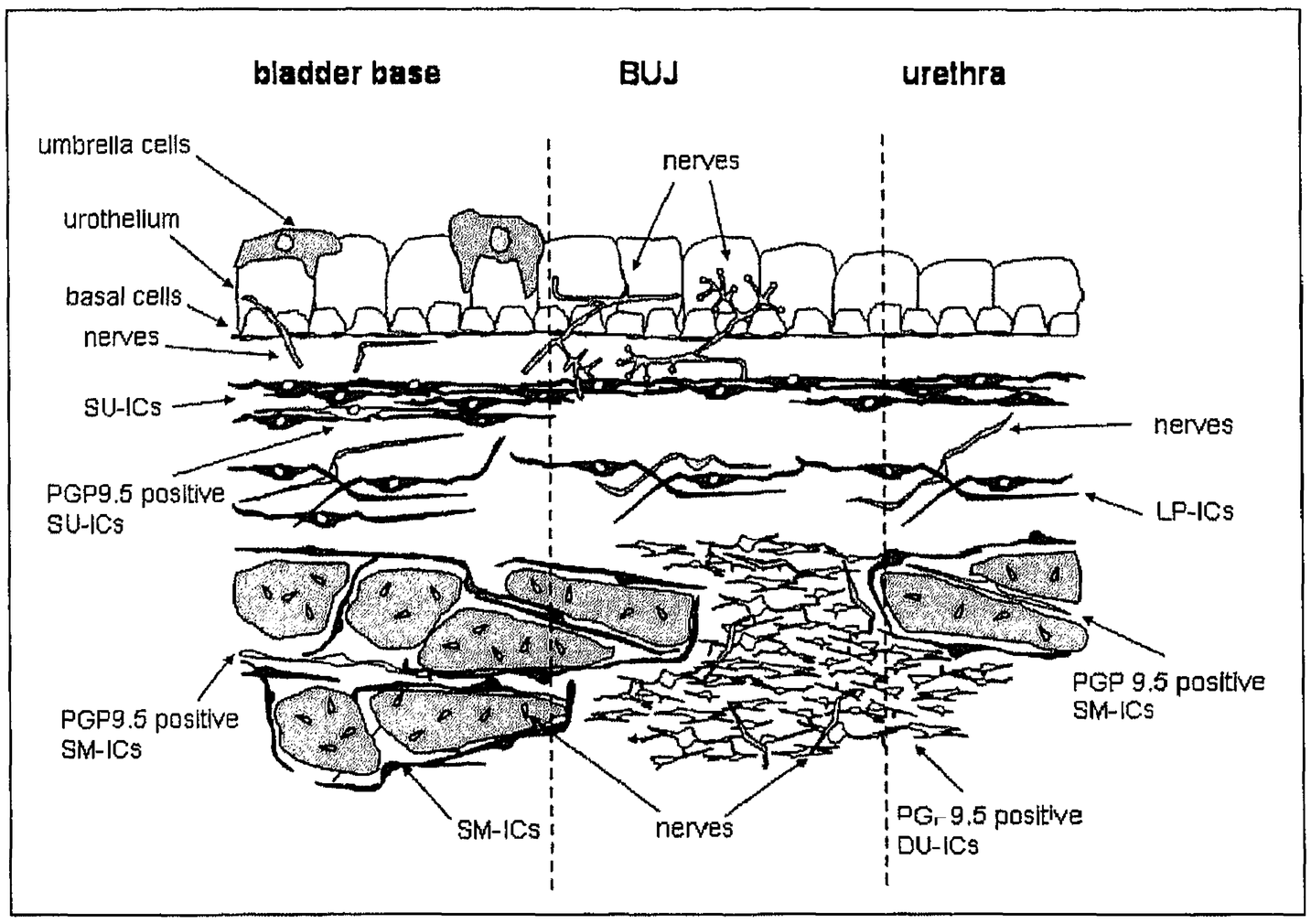

Figure 6. Diagrammatic representation of the cellular components of the bladder wall in the region of the bladder base, the bladder urethral junction (BUJ) and the urethra. This cartoon shows a summary of the structural differences between the bladder base, the BUJ and the urethra. The urothelium of the bladder base contains some umbrella cells, while in the urothelium of the BUJ and the urethra no umbrella cells could be identified. The thickness of the urothelium is less in the urethra compared to the BUJ and bladder base. In the BUJ there is a dense nerve innervation in the urothelium, while in the bladder base only sparse nerve innervation can be seen and in the urethra there is an absence of nerve innervation in the urothelium. Some of the urothelial nerves in the BUJ have axons with varicosities, which might indicate the release of substances by these nerves. Some of the sub-urothelial interstitial cells (SU-ICs) of the bladder base also stain for the nerve marker PGP9.5, while this co-localisation is not found in the BUJ and urethra. In the BUJ there seems to be less SU-ICs. Some the urothelial nerves in the bladder base and BUJ penetrate the sub-urothelium. Some axons of the urothelial nerves in the BUJ that penetrate the urothelium have varicosities. The sub-urothelial axons of these nerves lie in close relation with the SU-ICs. Below the SU-ICs the lamina propria ICs and nerves are shown. There is no co-localisation of PGP9.5 and vimentin in this layer. Below the lamina propria in the bladder base and urethra the muscle can be seen. Small nerves lie in the muscle bundles while around the muscle bundles the surface muscle interstitial cells (SM-ICs) can be seen. Note that some of these SM-ICs are PGP9.5 positive. In the area between the BUJ and the urethra there is an absence of muscle bundles. This area contains a large amount of interstitial cells; deep urethral interstitial cells (DU-ICs). These DU-ICs show co-localisation of vimentin and PGP9.5 and lie in close relation with nerves.

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Chapter 4

Ubiquitin hydrolase (PGP 9.5) in the obstructed bladder: evidence for tissue remodelling involving a sub-set of interstitial cells

S Grol, PBM Essers, GA van Koeveringe, J de Vente and JI Gillespie

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Abstract

Aim The aim of this study was to examine the expression of ubiquitin hydrolase (UBH), an enzyme which is part of the ubiquitin-proteasome system involved in the regulation of cell growth and differentiation, in order to gain insight into the cell types and processes underlying the tissue remodelling that occur following bladder neck damage.

Methods Three groups of male guinea pigs were used: control (not operated, n=4), sham (n=5) and obstructed (n=6). Bladder outflow obstruction was achieved by implanting a silver ring around the urethra which was left in situ for 2-4 weeks. Sham operated animals underwent the same operative procedure but no ring was implanted. The bladders were removed and incubated in Krebs solution at 36°C, gassed with 95% O_2 and 5% CO_2, Tissues were then fixed in 4% depolymerised paraformaldehyde and processed for immunohistochemistry. We used antibodies raised against ubiquitin hydrolase (UBH)/protein gene product 9.5, cyclo-oxygenase type I and vimentin. Specific antibody binding was visualised using the appropriate secondary antibodies.

Results Staining with an antibody to UBH revealed the presence of both sensory and motor nerves in control, sham and obstructed bladders. In the control bladders this was the predominant staining pattern. In the sham and obstructed bladders UBH staining revealed additional positive cell types: cells associated with the outermost layers of the urothelium (the umbrella cells), in the lamina propria (the lamina propria interstitial cells (LP-ICs), on the surface of the muscle bundles (surface muscle interstitial cells; SM-ICs) and on the serosal surface (muscle coat interstitial cells; MC-ICs). All interstitial cells stained with vimentin. The interstitial cells within the muscle bundles (intra-muscular interstitial cells; IM-ICs) did not stain with UBH. The number and density of the UBH positive cells was greater in the obstructed compared to the sham suggesting a change in relation to the severity of damage to the bladder neck.

Conclusion The expression of UBH implies the retargeting of proteins marked for degradation in the proteasome. Increased expression of UBH in the LP-ICs and SM-ICs demonstrates that these cells are active in the early and late stages of the tissue remodelling resulting from obstruction. These results demonstrate a further subset of interstitial cells which might be involved in the increased deposition of extracellular material and tissue remodelling.

Introduction

There has been a great deal of interest in the heterogeneous population of cells in the bladder described as interstitial cells. They are found in all species examined so far (mouse [1, 2], rat [3], guinea pig [4-6] and human [4, 7, 8]) and are likely to be involved in numerous, varied key regulatory systems in the bladder. In the guinea pig bladder, cells described as interstitial cells are found below the urothelium, within the lamina propria, surrounding the muscle bundles, lying with the muscle bundles and on the outer coat of the bladder [4, 5, 9-11]. Several criteria have been used to identify and distinguish these cells from each other and it is now accepted that there are different interstitial cell types. For example, one population of cells appear to express the stem cells factor receptor cKit [6]. The sub-urothelial interstitial cells SU-ICs) respond to nitric oxide (NO) with a rise in cGMP while those in the lamina propria do not [4, 5]. Also, the surface muscle interstitial cells (SM-ICs) associated with the inner muscle layers express cyclo-oxygenase I (COX I) while those in the outer muscle layers do not [11]. Despite a growing appreciation of the types and sub-types of bladder interstitial cells we know very little about the functions they might sub-serve. For one sub-type, the SM-ICs, it has been suggested that they interact with the smooth muscle regulating excitability and contraction [2, 12]. However, the role of the other sub-types of interstitial cells remains an enigma.

During bladder outflow obstruction there are many structural and functional changes in the bladder wall: the smooth muscle is more active and sensitive to acetylcholine [13-15], the urothelium thickens, the smooth muscles hypertrophy and there is a punctuate loss of the motor nerves. In addition there is an increase in number of muscle interstitial cells [16, 17] It has recently been suggested that these changes are triggered not by the obstruction *per se* but by the local damage that occurs to the bladder neck following surgery and which can be exacerbated by physical obstruction [15]. Such local damage results in global changes in the bladder wall. The mechanisms generating and coordinating the global changes from damage in a specific location are not known.

The protein, protein gene product 9.5 (PGP 9.5), is a small protein (27kD) which was originally isolated from brain [18, 19]. This molecule is now known to be a ubiquitin COOH-terminal hydrolase (UBH) and forms part of a complex ubiquitin-proteasome system. The binding of ubiquitin regulates and modulates protein function. The proteasome system is a non-lysosomal proteolytic pathway regulating degradation of proteins involved in cell growth, modulation of specific membrane receptors and rearrangement of cytoskeletal elements [20-22]. UBH separates ubiquitin from its protein substrate affecting the targeting of the protein and thus functions also in a salvage pathway.

It was originally thought that UBH was confined to neurones and neuro-endocrine cells [19]. However, further studies have located UBH in a number of different cells types [23, 24]. UBH is no longer considered to be a specific marker for neurones. Rather, it is an indicator of cells undergoing major

changes in structure, differentiation and phenotype. It may also be involved in the regulation of the early stages of inflammation [25].
In studies of nerve distributions in obstructed bladder it was recognised that cells other than neurones were staining with UBH. This paper describes these non-neural cells, identifying them as a sub-set of bladder interstitial cells.

Materials and Methods

Three groups of male guinea pigs were used: control (not operated, n=4), sham (n=5) and obstructed (n=6): Hartley strain guinea pigs, weighing between 266-299 grams, were housed in a temperature and light (12h light/dark cycle) controlled room and allowed free access to food and water. The institutional animal care and use committee of Maastricht University approved all the animal experiments conducted in this study. All procedures were in line with the EC guidelines.
Surgical procedure: The procedures used were similar to those reported previously [26]. Briefly, in 6 animals a partial outflow obstruction was induced while 5 underwent sham operation. Animals were sedated with ketamine (40 mg/kg) and xylazine (3 mg/kg) administered intraperitoneally. Once sedation was achieved and the animals were prepared under sterile conditions, the bladder neck and urethra were exposed via a vertical midline abdominal incision. A silver jeweler's jump ring (1.8 mm internal diameter) was placed loosely around the proximal urethra. Bladder outlet obstruction occurred during subsequent growth of the animal. The analgesic medication flunixinum (5mg/kg s.c.) and the antibiotic gentamicin (5mg/kg i.m.) were administered once postoperatively. Sham surgery was performed in an identical manner, except for inserting the ring around the urethra.
Four weeks after the operation, the weight gain in the contol group was 200 $\pm$ 65, in the sham operated animals it was 150 $\pm$ 33 grams compared to the animals with a bladder outflow obstruction 100 $\pm$ 53 grams.
After 4 weeks, the guinea pigs were killed by cervical dislocation. The urinary bladder was removed and placed in ice-cold Krebs solution containing 121.1 mM NaCl, 1.87 mM KCl, 1.2 mM CaCl2, 1.15 mM MgSO4, 25 mM NaHCO3, 1.17 mM KH2PO4, 11.0 mM glucose, bubbled with 5% CO2 and 95% O2 (pH 7.4). Each bladder was divided in two pieces, a ventral piece and a dorsal piece, and maintained in Kreb's solution containing 1mM of the non-specific phosphodiesterase inhibitor isobutyl-methyl-xanthine (IBMX: Sigma-Aldrich) at 36°C for 30 minutes. Incubations were terminated by immersing bladder pieces in ice-cold fixative solution of 4% freshly prepared depolymerised paraformaldehyde for 120 min at 4°C. Then, tissues were washed at 4 °C in 0.1M phosphate buffer containing 10% sucrose (24 h), 20% sucrose (24h) and 30% sucrose (24h). The tissues from the lateral wall were then snap-frozen with CO_2 in Tissue-Tek O.C.T. compound to form a single block. Cryostat sections (10 µm) were cut, such that each section was perpendicular to the urothelial

surface. Sections were then thawed on to chrome-alumn-gelatin-coated slides and processed for immunohistochemistry.

Immunohistochemistry : Sections were dried for 60 min at RT followed by three washes with Tris-buffered saline (TBS; pH 7.6), and thereafter incubated overnight with primary antibodies at 4 °C. The mouse antibody against vimentin (Sigma-Aldrich) was diluted 1:5000. To visualize UBH we used rabbit anti-PGP9.5 (1: 2000; Abd Serotec); the selectivity and an estimate of the detection limit of these antibodies have been described previously [27-31]. After overnight incubation with the primary antibodies diluted in TBS containing 0.3% (v/v) Triton X-100 (TBS-T), sections were washed in TBS, TBS-T and TBS; each wash step lasted 15 min. Rabbit primary antibody was visualized using Alexa Fluor 488 donkey anti-rabbit IgG (H+L) conjugate (Molecular Probes), diluted 1: 100 in TBS-T. Mouse primary antibody was visualized with Alexa Fluor 594 donkey anti-mouse IgG conjugate (Molecular Probes), diluted 1:100. Sections were incubated with the secondary antibodies for 90 min at RT in the dark. After washing with TBS-T, and TBS, sections were mounted with TBS-glycerol (80%). Typically, for each bladder the staining was done in duplicate and repeated at least on 2 separate days. Only the lateral wall was studied and all observations were accumulated from the different slides and from the different bladders.

Sections were analysed and photographed using an Olympus AX70 microscope using a x4, x10, x20 and x40 (oil) objective. For the detection of Alexa 488 fluorescence we used a narrow band-pass MNIBA-filter and for the detection of Alexa 594 we used a filter with a narrow excitation band, the U-M41007A filter (both filters are from Chroma Technologies). The microscope was equipped with a cooled CCD Olympus Digital video camera F-view. Images were stored digitally as 16 bits images by using the computer program cell-P® (Olympus, Germany). The number of grey values was reduced using a linear function into 4095. Images were arranged with the program Adobe Photoshop 5.5 or 7.0.1 (San Jose, CA, USA).

Results

The major findings of this study are: in sham operated and obstructed bladders there is an increase in the expression of UBH compared to the lateral wall of control bladders (Figure 1). In the control bladders UBH was located to both sensory and motor nerves. Occasionally, UBH was found in the umbrella cells of the urothelium. In the lateral wall of the sham and obstructed bladders several differences were observed. There was a distinct thickening of the urothelium (Figure 1 B and C) with a more pronounced expression of UBH. Also, UBH positive cells were found in the lamina propria and on the surface of the muscle bundles. These cells were more abundant in the obstructed compared to the sham bladders. In addition, there was a profound thickening of the outer coat (muscle coat) of the bladder in sham and obstructed animals. The majority

of these cells were UBH positive. The following section describes these basic observations in greater detail.

In the lateral wall of the control bladder a few umbrella cells could be found which were strongly positive for UBH in the urothelium (Figure 2 A). Adjacent cells stained weakly or not at all. The cells lying immediately below the urothelium, the SU-ICs did not stain with UBH but were vimentin positive (Figure 2 A). In the lateral wall of the sham and obstructed bladders marked changes were noted in the urothelial layer. The number of cell layers forming the urothelium was in creased and the number of layers which were positive for UBH could be several layer thick (Figure 2 C). The vimentin positive SU-ICs layer was present in the sham and obstructed bladders but no UBH positive cells were detected (Figure 2 B and C).

Close examination of the lateral wall of the sham operated and obstructed bladders revealed that the basal and intermediate cell layers of the urothelium do not stain for UBH (Figure 2 B and C). These cell layers express the enzyme COX I (Figure 3) suggesting that the different cell layers of the urothelium sub-serve different functions.

Vimentin-positive interstitial cells are present in the bulk of the lamina propria between the SU-ICs and the muscle layer: the lamina propria interstitial cells (LP-ICs). In the lateral wall of the control bladder these cells were UBH negative (Figure 4 A). In the lateral wall of the sham bladders cells two populations of vimentin positive cells were now observed. The majority were UBH negative while there is a small population of UBH positive cells (Figure 4 B). In the lateral wall of the obstructed bladders the proportion of LP-ICs which were UBH positive was markedly increased (Figure 4 C).

One sub-type of interstitial cell is located on the surface of the muscle bundles (SM-ICs). In the lateral wall of the control bladders these cells are vimentin positive and do not express UBH (Figure 5 A), while in the lateral wall of the sham bladders there are occasional UBH positive cells (Figure 5 B). In the lateral wall of the obstructed bladders there was an increased number of SM-ICs and the majority of these were UBH positive (Figure 5 C). Figure 6 shows further examples illustrating the increased expression of UBH in the lateral wall of the sham and obstructed bladders. Importantly, another striking observation in the obstructed bladders was the presence of the cell bodies of the UBH positive cells in small clumps or nodes (Figure 6 C).

Within the muscle bundles there is a further population of interstitial cells (intra-muscular interstitial cells; IM-ICs). When these cells are examined in the lateral wall of the control, sham and obstructed bladders almost no cells express UBH (Figure 7). Interestingly, IM-ICs in the control, sham and obstructed bladders can be found which are in close proximity to UBH nerve fibres (Figure 8).

As shown in Figure 1 D, E and F the muscle coat becomes thicker in the lateral wall of the sham and obstructed bladders, with the obstructed bladder wall being thicker than that of sham-operated animals. This is illustated in more

detail in Figure 9. The muscle coat of the control bladders is only one cell layer thick. These cells show immunoreactivity for vimentin, identifying them as muscle coat interstitial cells (MC-ICs). These MC-ICs show no UBH-IR (Figure 9 A). In the sham operated and obstructed bladders the muscle coat MC-ICs are several cell layers thick (Figure 9 B and C), and the MC-ICs show immunoreactivity for both vimentin and UBH.

Discussion

The antibody to the protein PGP 9.5 has been used extensively as a non-specific marker of neurones and neuronal processes. In the guinea pig urinary bladder it is indeed an effective marker of nerve fibers and neuronal cells, i.e. ganglion cells. However, it has long been known that the protein PGP 9.5 is expressed in other cell types [24]. The realisation that PGP 9.5 was a member of a family of enzymes involved in protein de-ubiquitination (ubiquitin hydrolase; UBH) advanced our understanding of protein metabolism and management in cell function associated with proliferation and differentiation [21, 32]. The processes of protein targeting and catabolism are highly regulated processes [33]. Ubiquitin is a small 76 amino acid protein which is covalently inked to proteins by specific proteins (E1, E2 and E3) to alter their function [21, 32]. Several ubiquitin molecules can be linked together to produce the appropriate effect. Removal of these bound ubiquitin molecules occurs by a family of de-ubiquitination enzymes which alters the targeting of the proteins. The process of ubiquitination and de-ubiquitination enzymes in regulating protein function has been likened to the relationship between phosphatases and kinases [25, 34]. Thus, the up-regulation of UBH in LP-ICs and SM-ICs suggests that they have become more active. They appear to be one of the first cell types, apart from the urothelium (see below), to show activation following damage to the bladder neck. Thus, the appearance of UBH may be an early marker for the changes that occur in the bladder following bladder neck damage. De-ubiquitination has been linked to regulation of NO signalling via regulation of the beta-subunit of the guanylate cyclase [35] and to COX II activation [36]. Under these circumstances the regulation of ubiquitin binding and release is associated with early inflammation and its regulation [25]. cGMP and prostaglandins (PGs) are now recognised to be associated with bladder interstitial cells [4, 5, 9, 11]. Indeed, it may be that some of the bladder interstitial cells are involved in the initiation and regulation of the early stages of bladder inflammation following injury. It is now becoming clear from these observations that the LP-ICs and SM-ICs play a role in the changes in the bladder that follow injury. The present study supports previous observations that there are distinct sub-sets of interstitial cells in the bladder [5, 9, 11].

As was argued previously, it does not appear to be the obstruction to bladder outflow per se that is the trigger for this pathological change [15]. Rather, as the same changes occur in the sham operated animals, this suggested that the pathological trigger is more related to damage of the bladder neck [15]. Once

the bladder neck is damaged the bladder undergoes major global changes: increased deposition of extracellular matrix, muscle hypertrophy, localised denervation and hypertrophy of the urothelium. It is not known how such global changes are triggered or coordinated. The present data are suggestive of a possible mechanism. The LP-ICs and SM-ICs constitute a network of interstitial cells extending through out the bladder. These cells appear to be particularly activated following bladder neck damage. It is possible that an initial local activation could be rapidly propagated throughout the network resulting in activation of the entire network. Activation might then trigger changes to the other cellular system in the bladder wall resulting in the observed global pathology. It has been demonstrated that there is a sub-population of LP-ICs and SM-ICs that express the enzyme COX I [11]. The local release of prostaglandin may be involved in this system of propagation and activation.

Bladder neck damage is also associated with changes to autonomous activity in the isolated bladder [15]. The mechanisms generating this activity, controlling its pacemaker functions and facilitating its propagation through out the bladder are not known. It has been suggested that these functions involve interstitial cells. However the heterogeneity of the interstitial cell population makes it difficult to identify which sub-type underlies which function. In the mouse, the SM-ICs and IM-ICs may be involved in the regulation of smooth muscle activity [2], The physiological role of the other sub-types of interstitial cells (SU-ICs, LP-ICs, and MC-ICs) remains an enigma. The present observations showing that the LP-ICs and SM-ICs react to bladder neck damage with an increased expression of UBH suggest at least one possible function for this sub-set. The SM-ICs also show immunoreactivity for the muscarinic receptor M_3 (Grol *et al. 2008* accepted BJU int.). This points out that the SM-ICs are able to react on cholinergic stimulation, thus these cells are part of the motor sensory system.

Importantly, this study confirms that the SU-ICs and IM-ICs appear to be distinct populations. The SU-ICs express receptors to acetylcholine (muscarinic type 3, M_3), PGs (EP Type 2) [37] and atrial naturetic peptide (ANP) [38]. They also respond to NO with a rise in cGMP [4, 5]. Thus these cells appear to be able to integrate different signal inputs. The function of these cells remain unknown. The IM-ICs also appear to be different. Two populations have been identified: those which express the enzyme choline acetyltransferase and a population which does not [39]. The function of this population is not known but it has been suggested that they are in close contact with nerve fibres and the smooth muscle suggesting that they may be involved in regulating muscle activity [39-41].

We have demonstrated that the urothelium is dramatically changed following bladder neck damage. It would appear that it is the outer layers of the urothelium, the umbrella cells, and the cells immediately below the umbrella cells which are activated and show an up-regulation of UBH. These cells are different form those in the basal and intermediate layers which do not show

such changes, identifying again distinct sub-types of epithelial cells within the urothelium. Thus, there appears to be a high degree of complexity in the urothelium. The function and physiological systems that this complexity sub-serves is not clear at present. However, the production of numerous signals (NO [42], adenosine trisphosphate (ATP) [43], acetylcholine [44, 45], and PGs [46]) suggests the potential for complex interactions and signalling in this region of the bladder [47]. One idea has been that the urothelium responds to increases in bladder volume by releasing NO, ATP and PG [47, 48]. These substances subsequently act upon sub-urothelial sensory nerves to influence bladder sensation [47, 49]. The alterations to the urothelium structure following bladder neck damage may therefore have profound effects on the output of these signals and their physiological effects. This may be one component of mechanisms contributing to the increased sensation in patients with pathological bladder changes.

The changes seen to the muscle coat with bladder neck damage are dramatic. Almost all of the cells in this system are activated to express UBH following severe bladder neck damage. In addition, these cells appear to undergo the highest degree of cell proliferation of any cell type in the pathological bladder. The function of this cell layer and the massive changes that are observed to occur remain unknown. They appear to be similar to the LP-ICs and SM-ICs in their expression of UBH but the high degree of proliferation might suggest that they have additional specific functions. Thus, they may form a further distinct sub-set of interstitial cells in the guinea pig bladder.

The clear heterogeneity of interstitial cells in the bladder generates specific problems when attempting to study isolated interstitial cells. If it cannot be demonstrated which sub-type of interstitial cell is being studied then we must be cautious about extrapolating the data obtained to specific functions. Indeed, without knowing what type of cell is being studied it is impossible to make connections to the integrated physiology of the bladder and its pathology.

In conclusion, the observations presented in this paper show what may be a novel sub-set of interstitial cells in the bladder wall of the guinea pig. The cells appear to form a network of activated and proliferating cells which extend throughout the bladder wall. More work is necessary to demonstrate the specific functions of these cells and whether they can alter underlying tissues and coordinate the global changes seen.

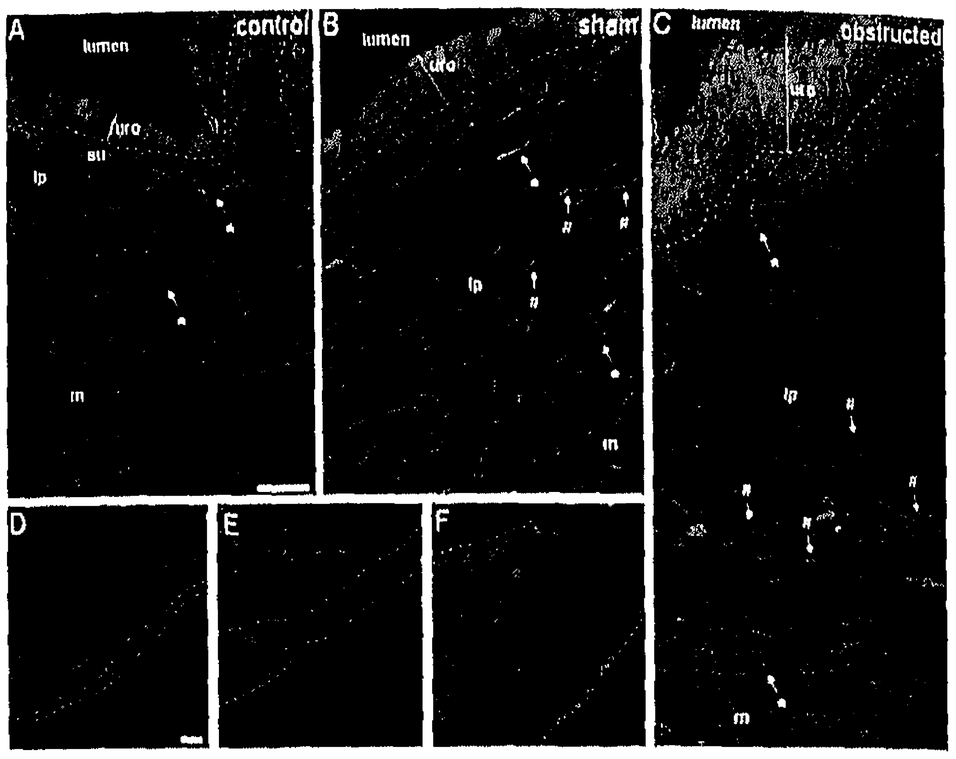

Figure 1. Structural differences in the guinea pig urinary bladder wall after bladder outlet obstruction. All panels are immunostained for the interstitial cell marker vimentin (vim, red) and ubiquitin hydrolase (UBH, green). Panel A shows an overview of the control bladder. Note the thin urothelium (u) and lamina propria (lp). The stars point to nerves in the lamina propria and muscle layers. Panel B shows an overview of the sham operated guinea pig bladder. Note the increase in thickness of the urothelium and lamina propria. A subset of the lamina propria interstitial cells (LP-ICs, #) shows immunoreactivity (IR) with UBH. The stars point to nerves in the muscle layer and lamina propria. Panel C shows an overview of the obstructed bladder. The urothelial cells become elongated. Note the further increase in thicknessof the urothelium and lamina propria. There is a further increase in the UBH-IR of the LP-ICs (#). The stars point to nerves in the muscle layer and lamina propria. Panels D, E and F show an overview of the muscle coat in the control (D), sham operated (E), and obstructed (F) bladder. In the control bladder (panel D) the muscle coat is one cell layer thick. These cells show IR for vimentin indicating that they are interstitial cells (muscle coat interstitial cells; MC-ICs). These MC-ICs are vimentin-positive and UBH-negative (vim+/UBH-). In the sham operated bladder (E) there is an increase in the number of MC-ICs. A subset of the MC-ICs are vim+/UBH+. While in the obstructed bladder all MC-ICs show IR for both vimentin and UBH. Calibration bar in A is for A, B and C; size 50 μm. Calibration bar in D is for D, E and F; size 20 μm.

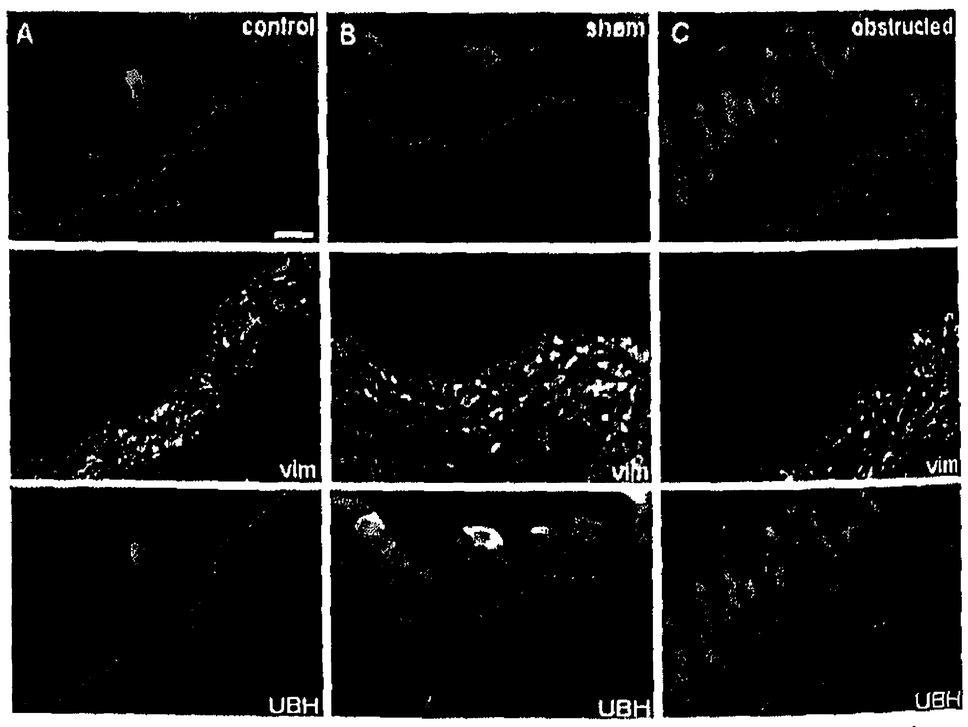

Figure 2. Upregulation of ubiquitin hydrolase (UBH) in the urothelium after outlet obstruction of the guinea pig urinary bladder. [illegible], B and C are immunostained for the interstitial cell marker vimentin (vim, red) and the UBH (green). The panels below show the original images of vim and UBH immunoreactivity (UBH-IR). Panel A shows the urothelium and sub-urothelium of a control bladder. Note that [illegible] the umbrella cells show UBH-IR. Panel B shows the urothelium and sub-urothelium of the sham operated bladder. There is an increase in UBH-IR in the urothelial cells. Panel C shows the urothelium and sub-urothelium of the obstructed bladder. Not only is there a further increase in UBH-IR in the urothelial cells visible, they are also shaped more elongated. Calibration bar in A is for A, B and C, size 20 μm.

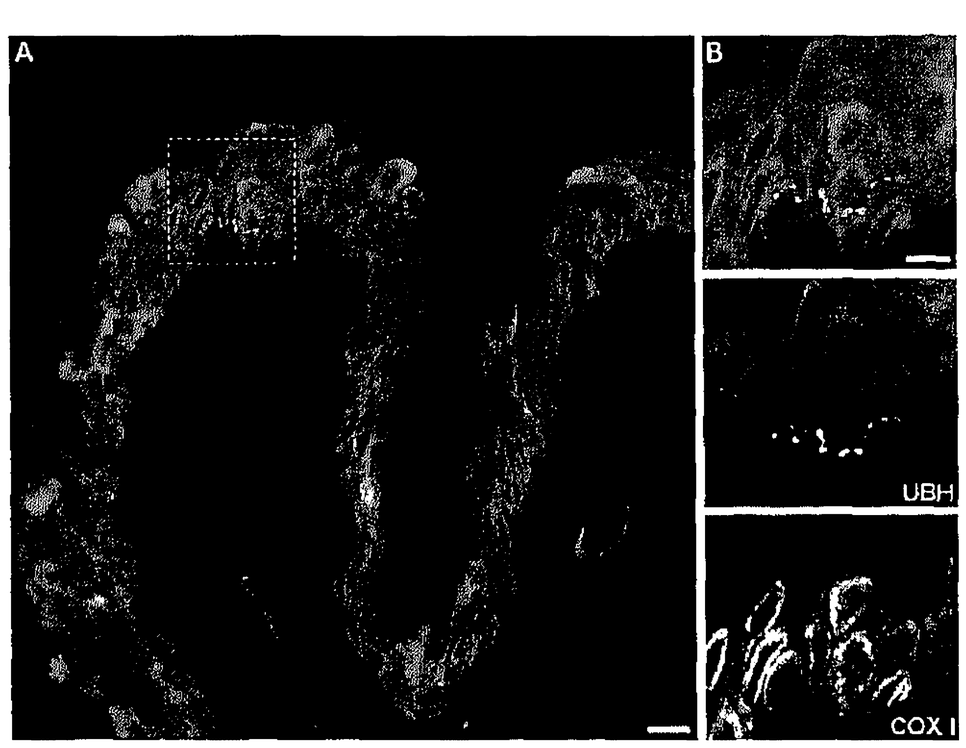

Figure 3. Distribution of ubiquitin hydrolase (UBH) and COX I in the urothelium of an obstructed guinea pig urinary bladder. Panel A and B are immunostained for UBH (green) and COX I (red). Panel A points out that the COX I-immunoreactivity (COX I-IR) is located in the basal urothelial cells while the UBH-immunoreactivity is located in the umbrella cells. Panel B shows a detail of panel A. The panels below panel B are the original pictures of which panel B is constructed. Calibration bars 20 μm in A and 10 μm in B.

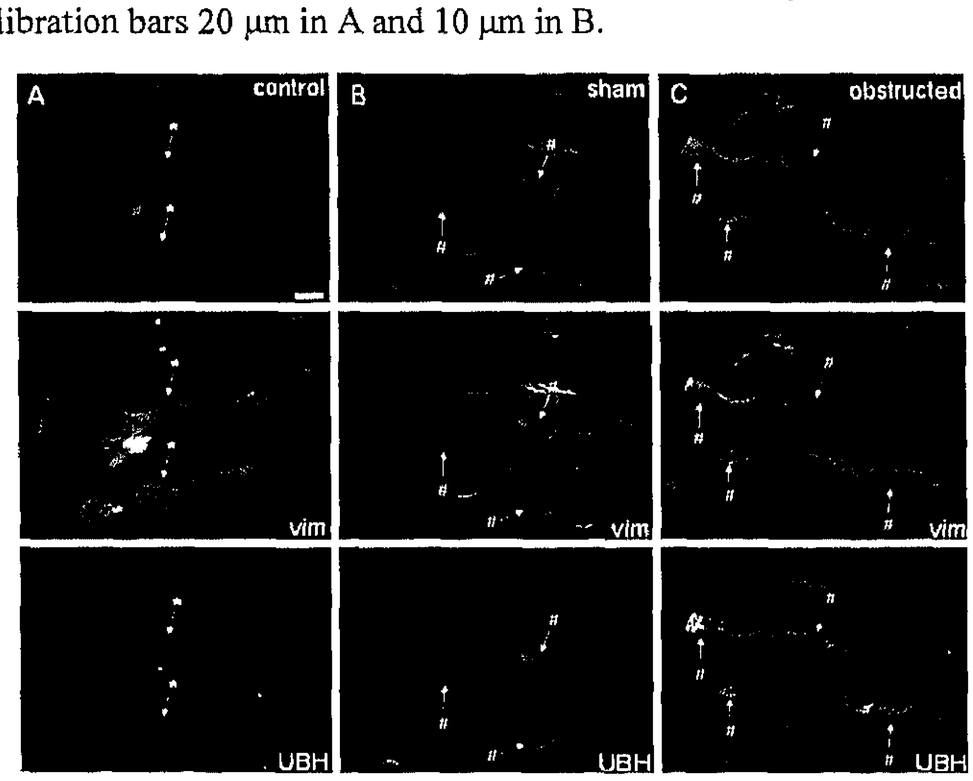

Figure 4. Upregulation of ubiquitin hydrolase (UBH) in lamina propria interstitial cells (LP-ICs) of the sham operated and obstructed guinea pig urinary bladder. Panels A, B and C are immunostained for UBH (green) and the interstitial cell marker vimentin (vim, red). The panels below show the original images of panel A, B and C. Panel A shows in detail the LP-ICs (*) of the control bladder. These cells do not show UBH-immunoreactivity (UBH-IR), while the LP-ICs of the sham operated bladder do show UBH-IR (panel B, #). Panel C shows the LP-ICs of the obstructed bladder. Note the upregulation of UBH in these cells and the increase in number of the LP-ICs. Calibration bar in A is for A, B and C; size 10 μm.

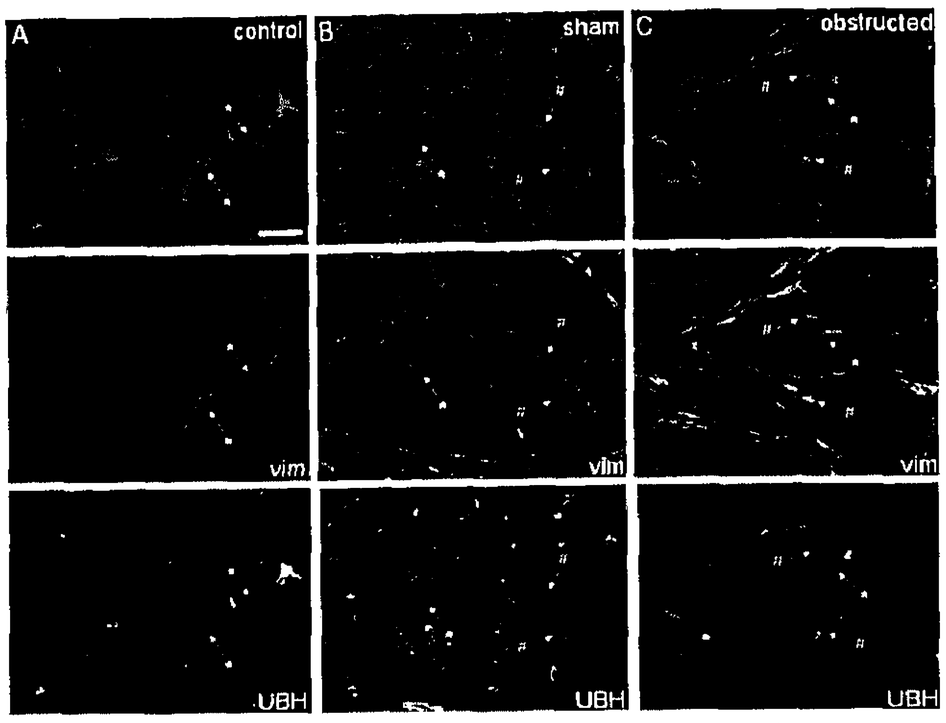

Figure 5. Upregulation of surface muscle interstitial cells (SM-ICs) and ubiquitin hydrolase-immunoreactivity (UBH-IR) in the sham operated and obstructed guinea pig urinary bladder. Panels A, B and C are all stained for ubiquitin hydrolase (UBH, green) and the interstitial cell marker vimentin (vim, red). The panels below show the original images of panels A, B and C. In the control bladder SM-ICs can be seen around the muscle bundles (panel A, *). These cells do not show UBH-IR. In the sham operated bladder there is an increase in number of the SM-ICs (panel B). Two types of SM-ICs can be identified; vim+/UBH- SM-ICs (*) and vim+/UBH+ SM-ICs (#). In the obstructed bladder there is a further increase in the number of SM-ICs (panel C). The two types of SM-ICs can also be seen here; vim+/UBH- SM-ICs (*) and vim+/UBH+ SM-ICs (#). Calibration bar in A is for A, B and C; size 10 μm.

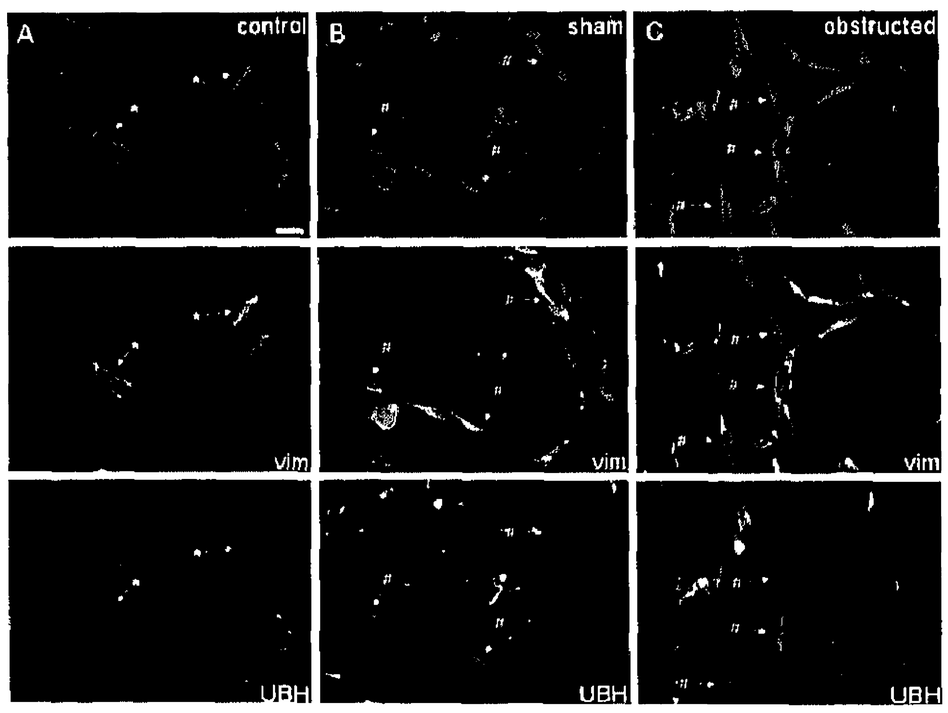

Figure 6. Upregulation of surface muscle interstitial cells (SM-ICs) and ubiquitin hydrolase immunoreactivity (UBH-IR) in the sham operated and obstructed guinea pig urinary bladder. Panels A, B and C are immunostained for ubiquitin hydrolase (UBH, green) and the interstitial cell marker vimentin (vim, red). The panels below show the original images of which panels A, B and C are constructed. In the control bladder there are SM-ICs visible around the muscle bundles A, *). These cells are vim+/UBH-. Panel B shows the sham operated bladder. Note the increase in number of SM-ICs. A subset of these cells shows UBH-IR (#). Panel C shows a detail of the obstructed bladder. Note the further increase in number of SM-ICs and upregulation of UBH-IR. Calibration bar in A is for A, B and C, size 10 μm.

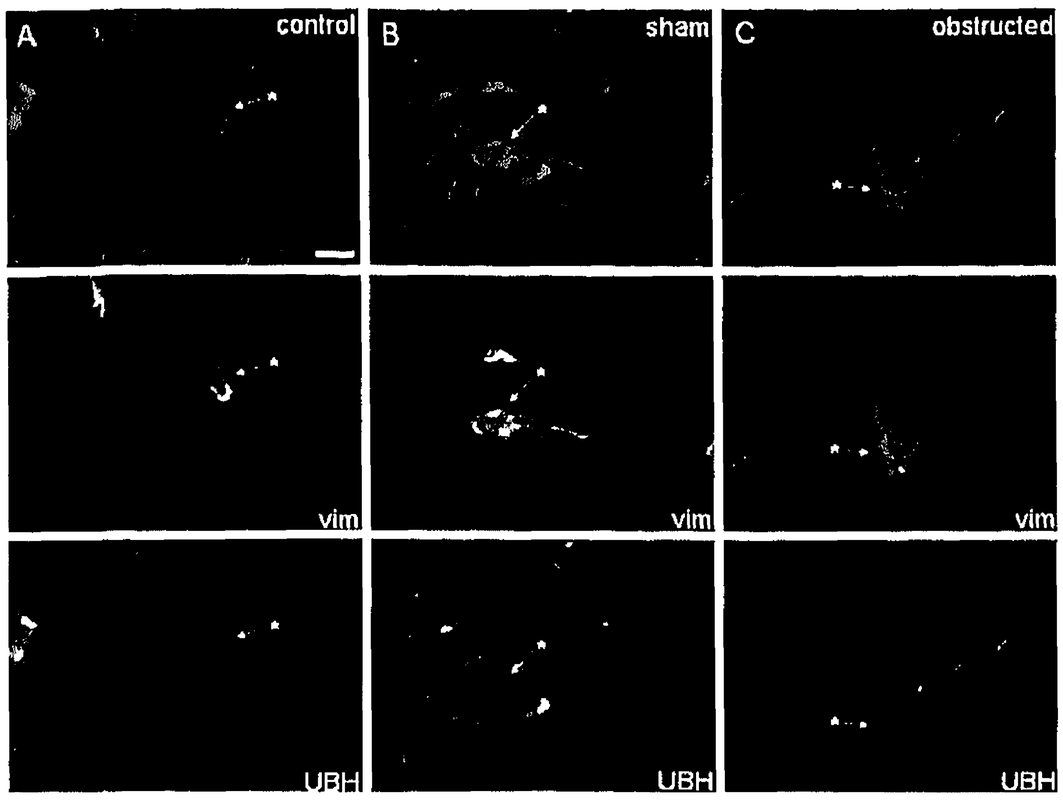

Figure 7. Intra-muscular interstitial cells (IM-ICs) do not change after outlet obstruction of the guinea pig urinary bladder. Panels A, B and C are all immunostained for ubiquitin hydrolase (UBH, green) and the interstitial cell marker vimentin (vim, red). The panels below show the original images of panels A, B and C. Panel A shows IM-ICs of a control bladder (*). Note that there is no UBH-IR. Panel B shows IM-ICs in the sham operated bladder (*). These cells are also UBH-. Panel Cshows IM-ICs in the obstructed bladder (*). No UBH-IR can be seen here. Calibration bar in A is for A, B and C, size 10 μm

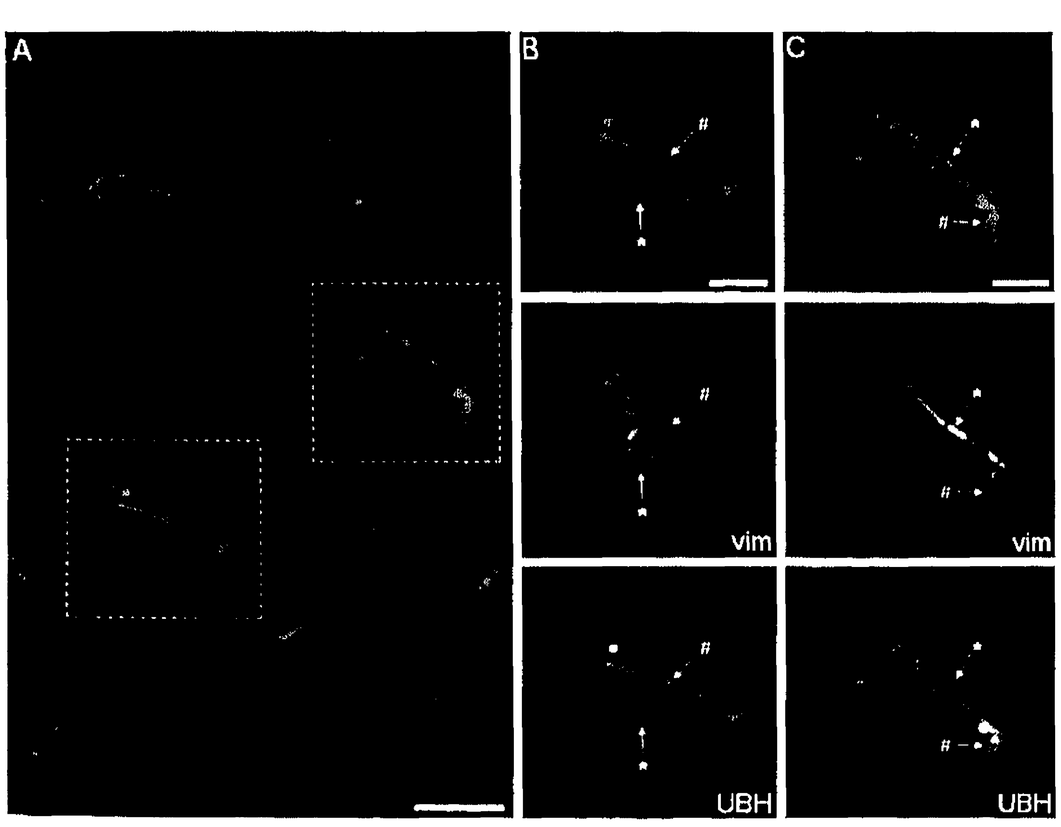

Figure 8. Intra-muscular interstitial cells (IM-ICs) are in close proximity with nerves. Panel A, B and C are stained with the interstitial cell marker vimentin (vim, red) and the enzyme ubiquitin hydrolase (UBH, green) which is located in nerve fibres. The panels below B and C show the original images of which panel B and C are constructed. Panel A shows an IM-ICs, fibres of IM-ICs and nerve fibres. Panel B and C show in more detail that the fibres of the IM-ICs (*) are in close proximity with nerve fibres (#). Calibration bars 10 μm in A, 5 μm in B and C.

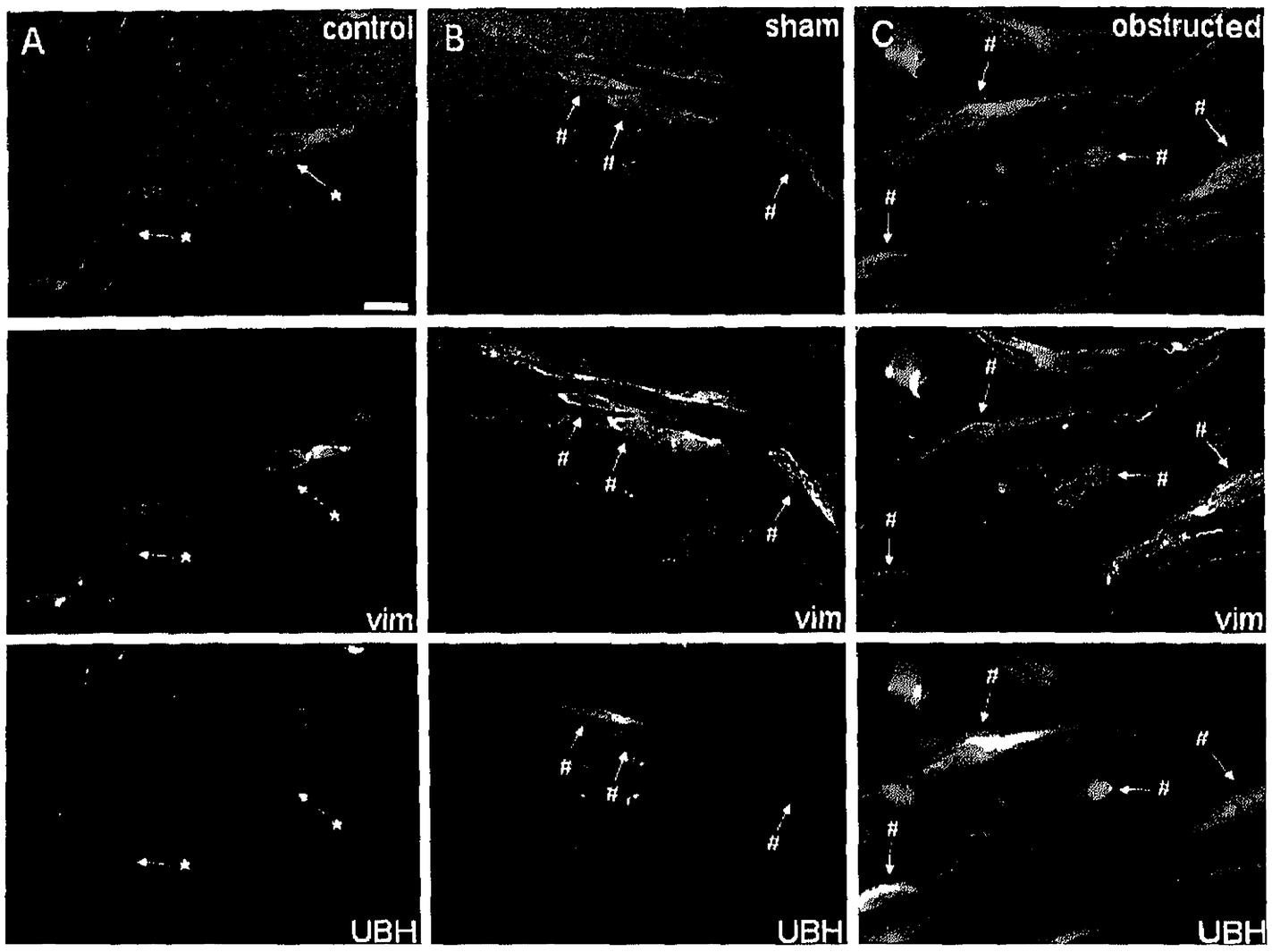

Figure 9. Upregulation of ubiquitin hydrolase immunoreactivity (UBH-IR) in the muscle coat interstitial cells (MC-ICs) of the sham operated and obstructed bladder. Panel A, B and C are all stained with the interstitial cell marker vimentin (vim, red) and ubiquitin hydrolase (UBH, green). The panels below show the original pictures of which panel A, B and C are constructed. Panel A shows the muscle coat of the control bladder. The MC-ICs (*) do not show any UBH-IR. Panel B shows the muscle coat of the sham operated bladder. There are now two types of MC-ICs; vim+/UBH- MC-ICs (*) and vim+/UBH+ MC-ICs (#). Note the increase in number of interstitial cells. Panel C shows a detail of the muscle coat of the obstructed bladder. All MC-ICs show UBH-IR. Note the further increase in number of MC-ICs. Calibration bar in A is for A, B and C, size 10 μm.

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Chapter 5

The localisation of cyclo-oxygenase immuno-reactivity (COX I-IR) to the urothelium and to interstitial cells in the bladder wall

R de Jongh, S Grol, GA van Koeveringe, PEV van Kerrebroeck, J de Vente and JI Gillespie

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Summary

Localised phasic contractions in the bladder wall (autonomous activity) have been hypothesized to be an integral part of a motor/sensory system contributing to bladder sensation. The sites responsible for generating this activity, the mechanisms involved in its propagation and modulation remain unknown. This phasic motor activity is modulated by exogenous prostaglandins. Therefore, analysis of the sites of prostaglandin production and action within the bladder wall may shed light on the mechanisms of generation and modulation of this phasic activity. In this paper we report the localization of immuno-reactivity indicative of the expression of cyclo-oxygenase enzyme type I (COX I-IR) within the bladder wall. Basically, three types of COX I-IR cell were identified: epithelial cells in the basal and intermediate layers of the urothelium, complex vimentin-positive and COX I-IR cells in the lamina propria and vimentin-negative COX I-IR cells in the lamina propria and on the surface of the inner muscle bundles. These vimentin-negative/COX I-IR cells appear to be in close apposition to a continuous network of vimentin-positive cells which extends from the lamina propria into the inner muscle layers and subsequently into the outer muscle layers. However, the interstitial cells in this region might form a distinctly different sub-type. Firstly, the interstitial cells in this region differ from those in the inner layer by their responsiveness to NO with a rise in cGMP. Two subtypes have been identified: cells on the surface of the muscle bundles and within the muscle bundles. Secondly, COX I-IR cells are not associated with the interstitial cells in the outer layers. The physiological significance for these apparent differences in the interstitial cell network is not clear. However, such differences are likely to reflect differences in the processes involved in their activation, modulation and control.

Introduction

Prostaglandins (PGs) are found in virtually all tissues where they exert a wide variety of functions including modulating smooth muscle activity, haemostasis and cytoprotection [1]. It has been known for nearly half a century that, in the bladder, PGs are released into the general circulation by the bladder in response to distension [2]. It has been established that the bladder PGs originate in both the urothelial and muscle layers [2-5]. The precise role of this endogenous PG is not known but it is well documented that exogenous PGs alters bladder activity both *in vivo* and *in vitro* influencing voiding and smooth muscle contractility. This occurs in different species: human [6-9], rat [10-12], guinea pig [13-15], rabbit [16-19] and monkey [20,21]. The underlying mechanisms which involve the PGs to alter voiding patterns and induce smooth muscle contraction are not known. Regarding PG induced changes in voiding frequency it was envisaged that they might act directly on the afferent nerves to modulate firing and so trigger micturition at lower bladder volumes [22-24]. With regard to a direct action on the muscle it was pointed out that they can be co-released with acetylcholine at efferent nerve endings and so directly contribute to muscle

excitation [20,21]. Alternatively, they might act indirectly on pre-synaptic motor terminals to affect the release of excitatory transmitters [11,12]. It was considered they might also inhibit acetylcholine esterase [9] or enhance myogenic bladder activity [6,22]. In other organs, PG production in fibroblasts resulted in a decrease in collagen production [25]. It has also been reported that prostaglandin production by fibroblasts plays a role in tumor necrosis [26]. In the intestine Powell *et all.* found that interstitial cells in the subepithelial space of the intestine are important in the organogenesis of the intestine [27], and secretion of prostaglandins by these interstitial cells is an important factor of this mechanism [27]. Therefore, it must be taken into consideration that, next to effects on contractility, PGs might have additional functions in the bladder, e.g. a role in cell proliferation [25].

Recently, a concept was proposed which attempts to integrate the effects of PG on the sensory elements of bladder control with its motor actions. Using the isolated bladder it was reported that exogenous PGs modulate the autonomous activity [28]. It has been argued previously that autonomous activity is part of a motor/sensory system operating within the bladder wall whereby localised contractions of the bladder wall stimulate firing in afferent nerves contributing to sensation [29,30]. PG induced modulation of the autonomous activity would thus increase bladder sensation and so modify voiding activity [28].

The origin of autonomous activity and how it is modulated by PGs is unknown. It has been hypothesized that it is generated within and distributed by a network of specialised cells in the bladder wall: interstitial cells [30,31]. The precise identification and definition of what is an interstitial cell in the bladder is, at present under discussion. The initial description of interstitial cells was based on their ability to show a rise in cGMP in response to nitric oxide donors [31,32]. Different sub-types of $cGMP^{+}$ interstitial cell have been identified lying principally in the sub-urothelial layer and in the outer muscle layer [31]. Finally, the stem cell factor receptor cKit, which identifies interstitial cells in the gut, has been reported to mark cells in the bladder [33]. However, this cKit staining is proving difficult to reproduce. Direct evidence for the physiological role of interstitial cells is, so far, limited and comes from experiments which show that nitric oxide (NO), which generates a rise in cGMP in the interstitial cells, also abolishes autonomous activity [34].

Since exogenous PG modulates autonomous activity [28] further insights into the origin of this activity and possible links to interstitial cells may be got from a detailed understanding of where PGs are synthesised and where they act in the bladder. PGs are synthesised by the two distinct enzymes: cyclo-oxygenase type I (COX I) and cyclo-oxygenase type II (COX II). COX I is associated with cell somata in the urothelium and with a network of cells running over the muscle bundles [28]. A detailed description of the different cell types expressing COX-I is not yet available. Therefore, the present experiments were done to characterise the cells expressing COX I in the normal bladder and to determine the relationship between these cells to the networks of interstitial cells. The

distribution and characteristics of COX II expression in the bladder is the subject of a separate paper [28].

Materials and Methods

Guinea pigs (7 male, weight 270-350 g) were killed by stunning and exsanguinations. The urinary bladder was removed and placed in ice-cold Kreb's solution containing 121.1 mM NaCl, 1.87 mM KCl, 1.2 mM $CaCl_2$, 1.15 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.17 mM KH_2PO_4, 11.0 mM glucose, bubbled with 5% CO_2 and 95% O_2. The bladder wall was cut into 3 or 4 sections from the base to the dome. The procedures for isolation, stimulation with the NO donor diethylamine-NONOate (DEANOl Sigma-ALdrich), and detection of cGMP-immunoreactivity were as described previously [28,31]. Preparation of the cryostat sections and immunocytochemical procedures were as described before [28,31]. Specificity studies on the sheep or rabbit anti-formaldehyde-fixed-cGMP antisera have been published before [31] and preabsorbtion studies to ascertain the specificity of the COX I antiserum have been published before also [28]. Primary antibodies used were goat polyclonal antibody to COX I (1:2000; Santa Cruz Biotech, Santa Cruz, Ca, USA), rabbit anti-nNOS antiserum (1:3000, Diasorin), mouse anti-vimentin antiserum (1:5000; Sigma/Aldrich). Secondary antibodies were Alexa Fluor 488 donkey anti-sheep IgG (H+L) and donkey anti-mouse conjugate (Molecular Probes, 1:100); Alexa Fluor 594 donkey anti-rabbit IgG conjugate (Molecular probes, 1:100); CY3 donkey anti-goat IgG conjugate (Jackson Immunoresearch, Newmarket, UK; 1:800). Sections were analyzed using an Olympus AX70 fluorescence microscope, equipped with a narrow band-pass MNIBA-filter for the detection of Alexa 488, and for detection of CY3 and Alexa 594 we used a filter with a narrow excitation band, the U-M41007A filter (both from Chroma Technologies, Rockingham, Vt, USA). The microscope was equipped with a cooled charge-coupled device, the Olympus digital video camera F-view. Images were stored digitally as 16 bits images by using the computer program Cell^P (Soft Imaging Systems, Olympus, Germany). The number of grey values was reduced by using a linear function to 4095. Color images were produced by combination of the original grey values photographs using the Cell-P program. Images were arranged using the program Adobe Photoshop 7.0.1 (San Jose, Ca, USA) without further processing.

Results

The key basic observations regarding the distribution of COX I-IR in the bladder wall are illustrated in Figure 1. All animals showed the same results. Panel A shows a low power section of the entire bladder wall. COX I-IR cells are seen within the urothelium and also associated with a network of cells which extends from the lamina propria into the inner muscle layers. Note that there are fewer COX I-IR cells associated with the outer muscle layers. These

observations are illustrated in greater detail in panels 1 and 2 which show the sub-urothelial region (1) and outer muscle layer (2) at higher magnification.
COX I-IR is therefore associated with two distinct structures: the urothelium and a network of cells in the lamina propria and around the muscle. The patter of distribution of these network cells bears a strong resemblance to the distribution of vimentin positive (vim^+) cells in the muscle layers bladder wall but not the distribution of NO-responsive $cGMP^+$ interstitial cells (Figures 1 B and C). Note that the $cGMP^+$ interstitial cells are found in the outer muscle layers where there is considerably less COX I-IR. These observations on COX I-IR in the urothelium and associated with the networks of interstitial cells are described in more detail below.

COX I-IR in the urothelium and sub-urothelial layer

Figure 2 A illustrates a section showing the urothelium and sub-urothelial region, double stained for COX I (red) and cGMP (green). Figure 2 B shows a similar section but from a different bladder illustrating the combined colour image (top) and the component COX I (middle) and cGMP (lower) images. As reported previously, the umbrella cells lying on the surface of the urothelium and the sub-urothelial interstitial cell layer closes to the urothelium are sensitive to exogenous NO and demonstrate a rise in cGMP ($cGMP^+$). It is also clear that COX I-IR is associated with intense staining in the intermediate and basal layers of the urothelium (buc) and weekly in cells in the lamina propria (lamina propria interstitial cells (LP-ICs). Figure 2 B shows, in greater detail that, in this region of the bladder wall, the majority of the sub-urothelial $cGMP^+$ interstitial cells do not have COX I-IR.

The cells of the basal layer of the urothelium express neuronal nitric oxide synthase (nNOS) [31]. The relationship between these cells and those with COX I-IR is shown in Figure 3. In this experiment the sections were double stained with nNOS (green) and COX I (red). The nNOS positive ($nNOS^+$) cells are easily seen in the basal layer. These cells are also COX I-IR (showing green/yellow in the combined sections). Note that in the intermediate urothelial layer the cells are COX I-IR but not $nNOS^+$ (Figure 3 B). Thus, there are two types of COX I-IR cell in the urothelium: basal COX I-IR/$nNOS^+$ and intermediate COX I-IR/$nNOS^-$.

The layer of densely packed cells lying immediately below the urothelium, the sub-urothelial interstitial cells (SU-ICs) are responsive to NO with an elevation in cGMP [31]. Figure 4 illustrates a section double labelled to show these $cGMP^+$ cells (green) and the $vimentin^+$ cells (red). $cGMP^+$ umbrella cells are seen on the surface of the urothelium but there were no vim+ cells in any cell type within the urothelium. In contrast the cells immediately below the urothelium, the SU-ICs, were vim^+ and $cGMP^+$. Vimentin staining revealed a network of intracellular fibres while the cGMP staining was most intense within the cell bodies (Figure 4 B). Processes of these cells were seen to contain vim^+ fibres but these structures were often not strongly $cGMP^+$ indicating that there are intracellular gradients of cGMP. Vim^+ cells were seen in the layer of the

lamina propria between the SU-IC layer and the muscle layer (Figure 4 A). These cells did not demonstrate a cGMP signal and can be described as lamina propria interstitial cells (LP-ICs). Thus, there appear to be different types of interstitial cell within the lamina propria: a dense region of $cGMP^+/vim^+$ and sparse $cGMP^-/vim^+$ cells.

An additional feature of the the COX I/vimentin double staining becomes apparent when studying in detail the diffuse network of vim^+ cells in the region of the lamina propria immediately below the SU-ICs and above the muscle layer (Figure 5). These cells appear to form a distinct network of inter-connecting cells contiguous with the SU-ICs layer and with vim^+ cells within the muscle layer (see below). In this section no COX I-IR cells are visible within the SU-ICs layer. But, COX I-IR is seen within the cell bodies of the vim^+ LP-ICs (Figure 5 B).

Regions of the lateral wall, particularly towards the bladder base, could be found in which the density of LP-ICs was high (Figures 6 and 7). In these regions two different cell types were readily identified based on the expression of vimentin and COX I-IR. Figure 6A shows such a region at higher magnification. Small bipolar cells with round cell bodies which were COX I-IR but did not stain strongly for vimentin (*) (Figure 6B) and larger complex cells with multiple processes which showed COX I-IR and a diffuse network of vimentin fibres ((‡) and Figure 6 C) are now visible.

The network of vim^+ fibres was observed to continue from the lamina propria into the inner smooth muscle layer where they run primarily on the surface of the muscle bundles (Figure 7). Here, the vim^+ cells appear on the surface of the muscle bundles and so can be described as surface muscle interstitial cell (SM-ICs). Within this network cell bodies were apparent which were COX I-IR but that did not stain strongly for vimentin. Examples of such cells are shown in Figure 7 B and 7 C. At the junction of the lamina propria and inner smooth muscle cell layer small clusters of COX I-IR cell bodies were often observed (Figure 8). The cell bodies in these clusters did not stain strongly for vimentin but were in close proximity to vimentin positive cell processes (Figure 8 B and C). These collections of cell bodies which have the appearance of nodes were also apparent, although fewer in number and with fewer cells, laying between the muscle bundles of the inner muscle layer (see Figure 7A).

Outer muscle layers

The network of vim^+ SM-ICs was observed to extend into the outer muscle layers of the bladder wall (Figures 1 and 9). However, in this outer region there are few cell bodies which are COX I-IR. This points out that there must be different types of SM-ICs associated with the inner and outer muscle layers. This is supported in Figure 10 which shows sections double labelled for cGMP (green) and vimentin (red). As has been reported previously the SM-ICs of the outer muscle layers respond to NO with a rise in cGMP [31]. The cells bodies of these outer SM-ICs are clearly seen but these are not COX I-IR. In contrast, there are few $cGMP^+$ cells in the inner muscle layers (see also Figure 1). NO-

responsive cells producing cGMP are also found within the muscle bundles of the outer muscle layer: intramuscular interstitial cells (IM-ICs). Figure 10 illustrates that these IM-ICs are also vim^+.

Thus, these data support the idea that there are different sub-types of interstitial cell associated with the lamina propria and inter-muscular spaces in the guinea pig bladder. The different cell types which have been identified and described above, based on the staining for cGMP, vimentin, COX I and nNOS, are summarised in Table 1.

Discussion

It has been known for over 30 years that PGs are released from the bladder in response to stretch, the PGs coming from both the urothelium and muscle layers [19,24,35,36]. Sprem *et al.* found that intravesically administrated ketoprofen, a non-selective COX inhibitor, reduced detrusor instability [37]. Based on these findings it has been argued that the PGs play central roles not only in bladder physiology but also in the generation of bladder patho-physiology [2-5,38-40], this led to the trial of cyclo-oxygenase inhibitors for the treatment of bladder over activity [37,41]. One indication as to the possible role of PGs has come from experiments involving the infusion of PG into the bladder lumen. When this is done it gives rise to an increase in micturition frequency and the incidence of non-voiding phasic contractions between voiding episodes [42,43]. Prostaglandin production in the bladder serves also other functions. Bachteeva *et al.* reported that PGs play a role in the osmotic water permeability of the frog urinary bladder [44]. Several subtypes of prostaglandin receptors have been found in the bladder. It has been reported that the urothelium contains both the prostaglandin receptor subtype E2 (EP2), which plays a role in osmoregulation [45], as well as the EP1 receptor, which plays a role in the micturation reflex [46]. Schröder *et al.* reported that the EP1 receptor has a role in the development of detrusor overactivity caused by PGE2 and outlet obstruction [47]. The details of the mechanisms underlying this action of PGs on voiding frequency are not known.

Several possible mechanisms have been put forward. One idea is that the PGs have a direct effect on bladder afferent nerve fibres [48]. By increasing afferent nerve activity this would result in a more frequent activation of the micturition reflex. It is well documented that PGs cause a sensitisation of cutaneous nociceptors [49]. This might also occur in the bladder. Support for the idea comes indirectly from experiments in which the bladder was treated with capsaicin to remove the afferent contribution of C fibres. After functionally removing the C fibres the PG induced increase in micturition frequency of micturition was reduced [40]. A different mechanism involving an indirect action of PGs on afferent nerves has recently been proposed. It is known that small localised contractions occur in the bladder wall of many species. This complex activity is hypothesized to be the motor component of a motor/sensory system involved in the generation of afferent firing and bladder sensation

[29,31]. Using the isolated guinea pig bladder is has been shown that PGs increase the frequency of this phasic motor activity [28]. Thus, the increased phasic motor activity could result in an increase in afferent discharges and in so doing influence the point at which voiding is triggered [28].

As discussed above PGs are synthesised within the lamina propria and muscle layers. The present observations extend this broad observation and demonstrate specifically that the expression of COX I-IR predominates within two general cell systems in the bladder wall: (i) cells within the basal and intermediate layers of the urothelium and (ii) within a population of small cells which are closely associated with a network of vimentin positive cells. These vimentin positive cells are present throughout the sub-urothelial space of the lamina propria and extend over the surface of the muscle bundles which make up the inner layers. It is interesting and important to note that no COX I-IR was seen within the muscles indicating that, in the guinea pig, it is not the smooth muscle that is producing PG but the cells associated with the vimentin network.

These observations raise intriguing questions regarding the mechanisms of action of PGs in the guinea pig bladder. One hypothesis might be that the COX I in the basal urothelial cell layers which is activated by bladder distension. The PGs produced here could diffuse the relatively short distance to the sub-urothelial space in which sensory afferent nerve fibres are found. Thus, this arrangement would represent the site where there is a PG induced direct modulation of afferent nerves. However, the abundance of COX I-IR in the urothelium of the lateral wall and the relative paucity of afferent nerves indicates that this is not the only role for PGs produced by the urothelium in the lateral wall.

The present data also show that the COX I-IR cells in the base of the urothelium also express nNOS. Like PG, NO is known to be produced by the urothelium in response to stretch [50]. It is also known, on other cell systems, that PG production is influenced by NO and, conversely, that NO production is influenced by PGs [51,52]. Other signals also originate in the urothelium in response to stretch. Specifically ATP has been shown to be released [53] and one of its actions is to influence afferent nerve firing [54]. Also, there are reports that acetylcholine is released from the urothelium [55]. Thus, we conclude there is a complex and inter-related release of signalling substances from the urothelium in response to mechanical deformation.

To add to this complexity, the cell layer immediately below the urothelium, the sub-urothelial interstitial cell (SU-IC) layer can be a possible location for further integration of urothelial derived signals. These SU-ICs, in the guinea pig and human, respond to both exogenous and endogenous NO demonstrating a rise in cGMP [30,32]. These cells are also immuno-reactive for antibodies to the type 3 muscarinic receptor (M_3) (unpublished observation), purinergic receptor [56] and the type 2 prostaglandin receptor (EP2) (unpublished observation). Thus, the integrated output of PG, NO, ATP and cholinergic stimuli from the

urothelium can be further integrated and modulated on the SU-ICs. The specific function of the SU-ICs cells is, at present, not known.

Indeed, there is indirect support for such a possible integration on the sub-urothelial cells. The SU-ICs are in close relation with the network of interstitial cells of the lamina propria and on the surface of the inner muscles. The autonomous bladder hypothesis suggests that, based on the observations of Lagou *et al.* [34] that the muscle interstitial cells are involved in the activation and co-ordination of complex phasic activity (autonomous activity) within the smooth muscle [57] and it is this activity that is the motor component of the motor sensory system [30]. The amplitude and frequency of the autonomous activity is increased by cholinergic agonists [58,59] and ATP [60]. If this type of activity is generated within an interstitial cells network this points out that there are M_3/ATP activated pacemakers linked to a distributed network [59].

It has also been shown that bladder distension alters autonomous activity: an increase in bladder volume increases autonomous activity while a decrease in bladder volume inhibits it [61]. The complex scheme outlined above might provide an explanation for these volume related events. Mechanical deformation of the urothelium in the lateral wall triggers complex cascade of interacting signals within the urothelial epithelium resulting in the release of signals into the sub-urothelial space. There, these urothelial signals are further integrated on the SU-ICs. Activity in the SU-ICs is then distributed to the muscle via the vimentin positive interstitial cell network resulting in the excitatory and inhibitory effects on the phasic activity (see Figure 11).

Prostaglandin synthesis has been associated with the smooth muscle [24]. The precise cell types and their location have not been considered in detail, it was simply assumed that it originated in smooth muscle cells. The present observations put forward a new idea that, at least in the guinea pig, it is the COX I-IR cells associated with the network of vimentin positive interstitial cells on the surface of the muscle bundles which are responsible for synthesising PG in this layer. As already discussed one of the possible roles of this network of muscle interstitial cells is to generate and distribute signals leading to phasic contractions in the bladder wall [34,57,62]. Thus the micro-anatomical arrangement of cells in the muscle layer appears to involve COX I-IR (PG producing) cells contacting vimentin interstitial cells in close apposition to smooth muscle. Functionally, it can be hypothesized that this arrangement involves a PG regulation of activity in the vimentin network and consequently an input to the muscle. The actions of PG on the muscle would thus be indirect being interceded by the network of interstitial cells (see Figure 11).

The present observations also demonstrate a difference in the interstitial cells in the outer and inner muscle layers of the bladder wall, specifically the reduction in number of COX I-IR cells in the outer layers. Differences have been noted before [31,34]. However, the functional roles for these apparently different types of muscle interstitial cell are not known. What is clear is that the network of interstitial cells is complex and may sub-serve several different functions.

In conclusion, it is now quite clear that there are several complex signalling systems operating within the bladder wall. In addition, it is clear that these signals act within cells systems that interactions between the urothelium, interstitial cells, muscle, sensory fibres and intra-mural ganglia. It is a major challenge for the future to unravel this complexity not only in relation to the physiology and pharmacology of the bladder but also in relation to the origins and treatment of bladder pathology.

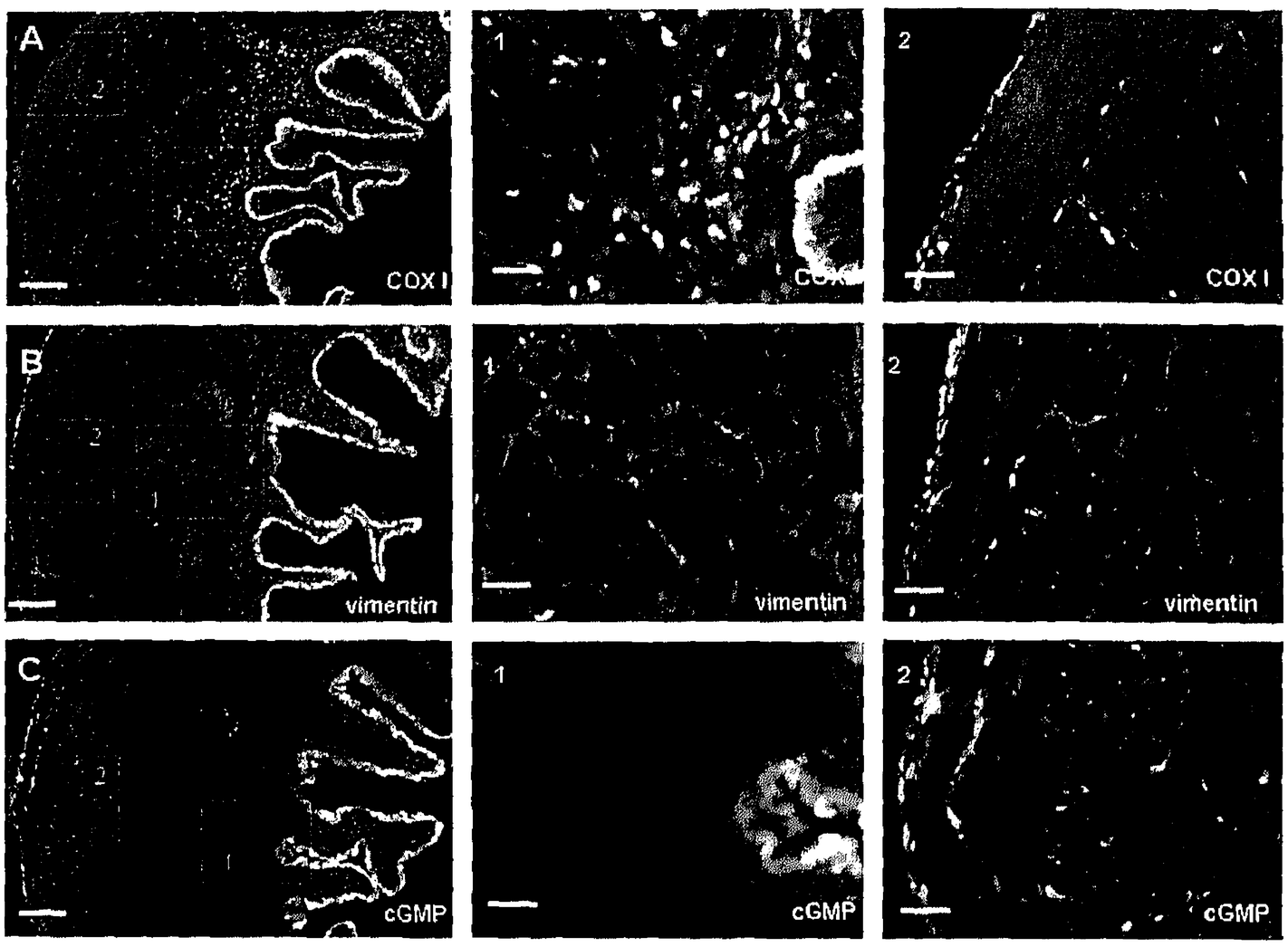


Figure 1. The localisation of COX I immunoreactivity, vimentin+ cells, and NO-responsive cGMP+ cells in the guinea pig bladder. A, B and C show respectively images stained with antibodies to COX I, vimentin and cGMP. In each panel two regions are identified (1 and 2) located in the outer and inner regions of the bladder wall. For A-C these identified regions are shown at greater magnification in the adjacent panels. A illustrates COX I-IR. More COX I-IR cells areare seen in the inner regions of the bladder. In B vimentin+ cells are seen in both the outer an inner layers. In C the presence of cGMP+ cells is clearly seen. No cGMP cells are seen associated with the inner muscle layer but the umbrella cells and sub-urothelial cells are cGMP+. Calibrations bars: 150 μm in A and 40 μm in B.

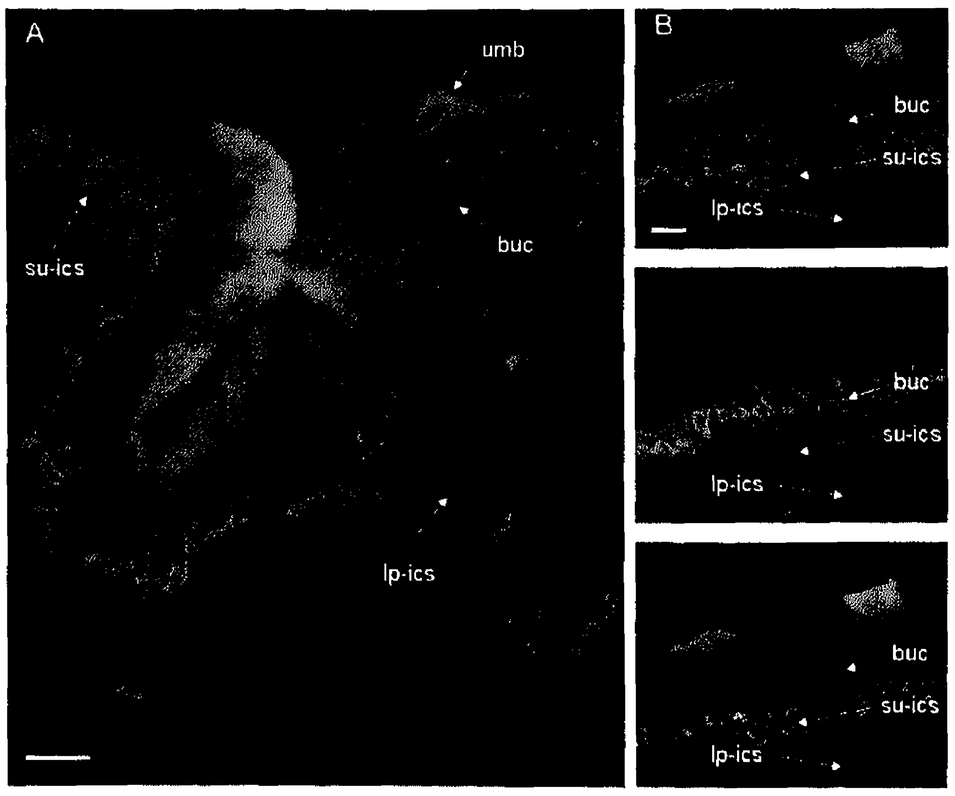
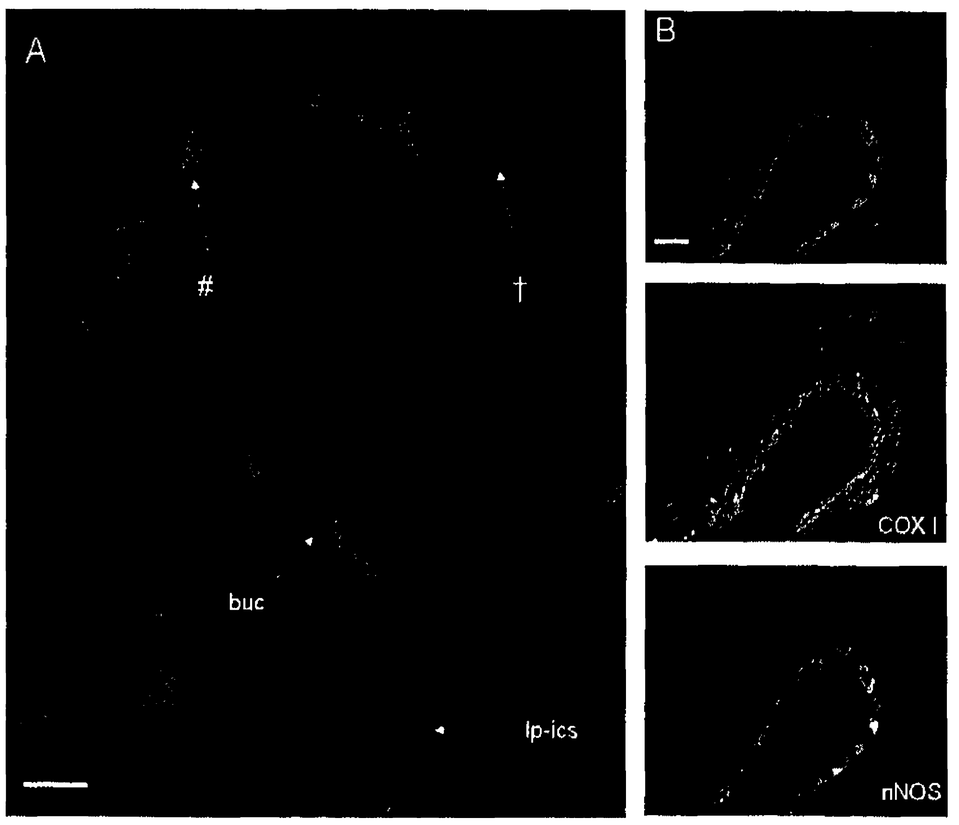


Figure 2. cGMP and COX I immunoreactivity (IR) associated with the urothelium in the guinea pig bladder. Panel A shows an image of the urothelium of the lateral wall stained for COX I (red) and cGMP (green). The preparation was stimulated with a NO donor to elevate cGMP levels in responsive cells prior to fixation. The umbrella cells on the surface of the urothelium (umb) and cells in the sub-urothelial space, the sub-urothelial interstitial cells (SU-ICs) are $cGMP^+$. The basal urothelial cells (buc) are stained intensely with the COX I antibody. In addition, COX I-IR cells are also found in the lamina propria interstitial cells (LP-ICs). B illustrates a section from a different bladder processed and stained in the same way as section A. The upper panel shows the combined colour image. The middle and lower panels show COX I-IR and cGMP-IR respectively. These two panels illustrate that the $cGMP^+$ SU-ICs are negative for COX I-IR. They also show that the COX I-IR cells in the buc are separated from the COX I-IR LP-ICs. Calibration bars 50 μm in A and 30 μm in B.



F gure 3. The dentif cation of cells expressing neuronal nitric oxide synthase (nNOS) and COX I immunoreactivity (IR) in the urothelial layer of the guinea pig bladder. Sections were co stained with antibodies to nNOS (green) and COX I (red). A shows an example of the staining pattern associated with the urothelium. $nNOS^+$ cells (#) are seen to be located within a single layer in the basal urothelium (buc). These cells also demonstrate COX I-IR. Note that the COX I-IR extends into further cell layers within the urothelium (†). B shows a further example from a different bladder. The combined image and the individually stained images are shown. The location of nNOS to a single layer in the basal urothelium is clearly seen while the more diffuse location of the COX I-IR is apparent. Calibration bars: 30 μm in A and B

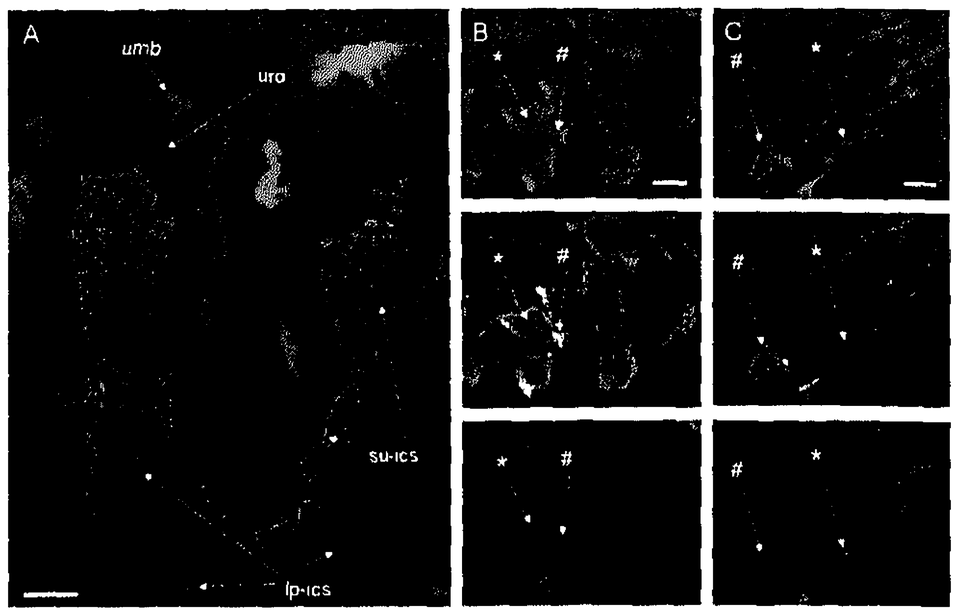


Figure 4. The location of vimentin$^+$ structures in the urothelium and sub-urothelial space. Sections were double labelled with antibodies to show cGMP (green) and vimentin (red). Panel A shows a section from the lateral wall of the bladder illustrating the urothelium and sub-urothelial space. The umbrella cells (umb) and sub-urothelial interstitial cells (SU-ICs) are cGMP$^+$. No vimentin$^+$ structures are seen within the urothelium. Vimentin$^+$ structures are seen in the sub-urothelial space. Intense staining is seen associated with the cells immediately below the urothelium while structures laying deeper within the lamina propria are vimentin$^+$ but more defuse. B and C illustrates examples of the cells in the sub-urothelial layer at higher magnification, showing the combined colour image (upper panel: cGMP-green, vimentin-red), the cGMP image alone (middle panel) and vimentin image alone (lower panel). The cGMP$^+$ sub-urothelial interstitial cells can readily be identified (*). Vimentin staining is associated with the cell bodies of these cells being highly concentrated around the cell nuclei (#). C illustrates a further feature. Vimentin$^+$ but cGMP$^-$ fibres are seen (# in C). Calibration bars 80 μm in A and 20 μm in B and C.

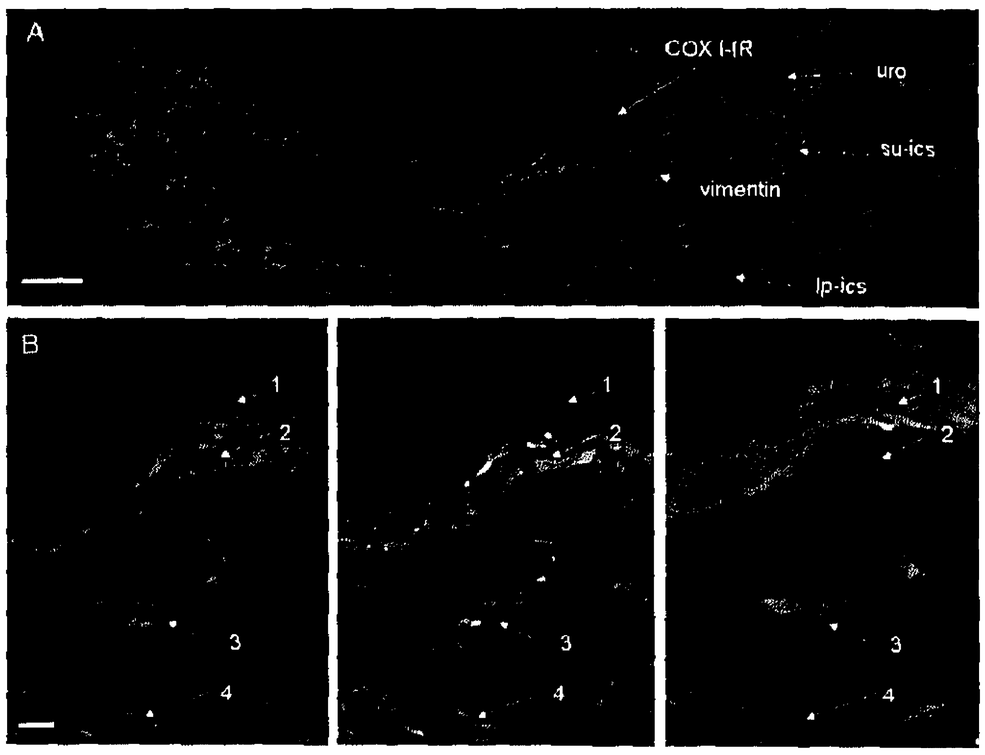


Figure 5. Identification of COX I immonoreactivity (IR) and vimentin in the lamina propria. Sections were double labelled with antibodies to COX I (red) and vimentin (green). A shows a low power image showing the urothelium, sub-urothelial layer, and the lamina propria. The COX I-IR cells are clearly visible within the urothelium (uro), the suburothelial interstitial cells (SU-ICs) and the lamina propria (LP-ICs). Below the SU-ICs there is a network of interstitial cells within the lamina propria (LP-ICs). Structures positive for vimentin and with COX I-IR are seen within this layer. B shows a section of the image in A at higher magnification. The panel on the left shows the doubled labelled image (vimentin-green, COX I-red), the middle panel shows the vimentin image and the right panel shows the COX-I image. The basal urothelial layer (1), sub-urothelial layer (2) are identified. The staining pattern of the vimentin$^+$ cells in the lp is highly suggestive for a network of vimentin$^+$ structures (4). In addition there appear to be COX I-IR cells (3).These cells are spindle shaped and are weakly positive for vimentin. They appear to lay on or between the vimentin$^+$ structures. Calibration bars 40 μm in A and 20 μm in B.

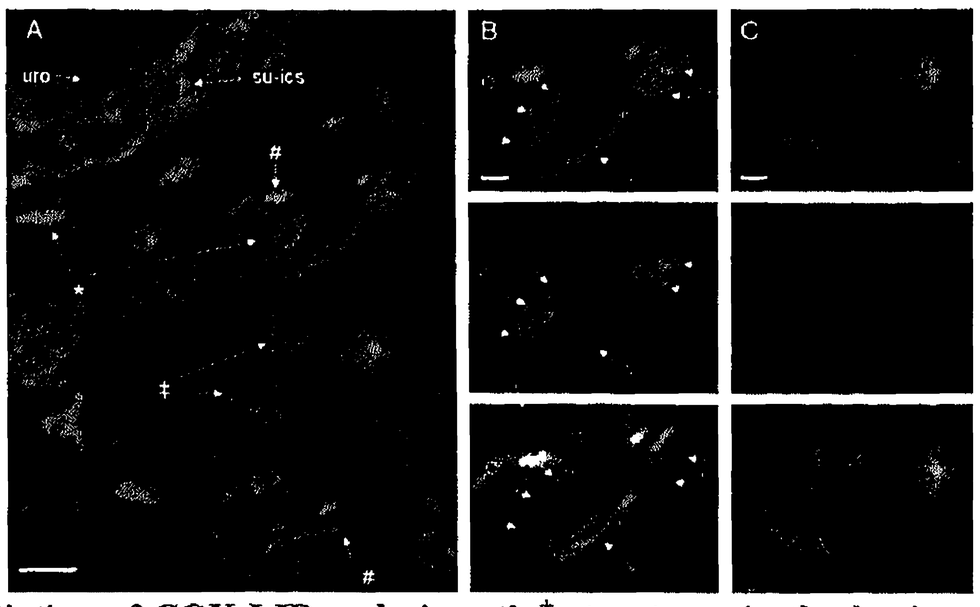


Figure 6. Differentiation of COX I-IR and vimentin$^+$ structures in the lamina propria. A shows a region of the lateral wall. The sections were stained with antibodies to COX I (red) and vimentin (green). A shows a region of the lamina propria immediately below the urothelium. The COX I-IR of the urothelium (uro) and spindle cells in the lamina propria are seen (*) (see also Figure 5). Also, cell processes expressing vimentin are visible. Large irregular cells are also seen which are COX I-IR and which have an extensive diffuse network of vimentin fibres (‡). B and C show selected areas of the image in A with the individual images of COX I (middle panel) and vimentin (lower panel). In B the COX I-IR cells are seen to have little or no vimentin staining. The edges of the COX I-IR cells are indicated by the arrows and (c). Vimentin$^+$ fibres are indicated (v). C illustrates the larger cells with the diffuse vimentin$^+$ network in the lamina propria. Calibration bars 20 μm in A, B and C.

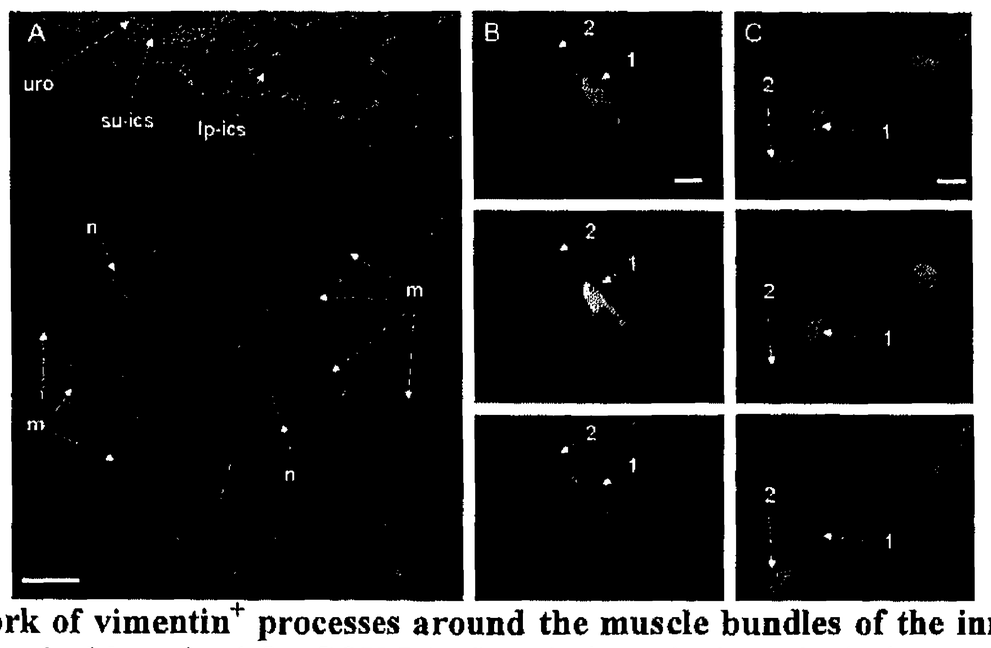


Figure 7. A network of vimentin$^+$ processes around the muscle bundles of the inner muscle layer. Sections were double stained for COX I (red) and vimentin (green). A shows a low power image. The COX I-IR cells in the urothelium (uro) and lamina propria (LP-ICs) are seen. The vimentin$^+$ fibres of the sub-urothelial cell layer (SU-ICs) and within the cell network in the lamina propria are also apparent. Muscle bundles in the inner muscle layer are shown (m). A shows a network of vimentin$^+$ fibres which are associated with the muscle bundles Collections of COX I-IR cells are seen between the muscle bundles (n). These structures are described as 'nodes'. B and C show examples of the COX I-IR cells and their relationship to the vimentin$^+$ fibres. B and C showing combined colour image (upper panel: vimentin-green, COX I-red), the COX I image alone (middle panel) and the vimentin image alone (lower panel). The COX I-IR cells (1) are weakly vimentin$^+$ and are associated with the vimentin$^+$ processes (2) of other cells. Calibration bars 240 μm in A and 10 μm in B and C.

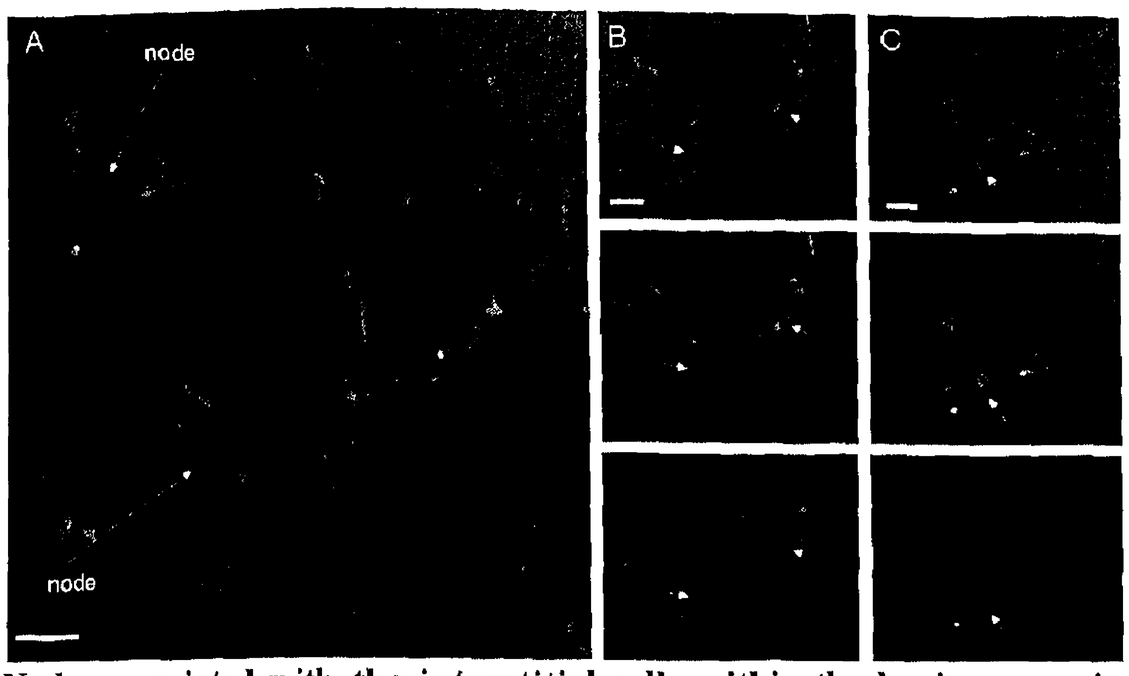


Figure 8. Nodes associated with the interstitial cells within the lamina propria and inner muscle layer. Sections were double stained with antibodies COX I (red) and vimentin (green). A shows a region of the lamina propria. Collections of COX I immonoreactivity (IR) structures (nodes) are seen associated with collections of vimentin$^+$ fibres. B and C show selected areas of the image in A with the associated individual images to COX I (middle panel) and vimentin (lower panel). The COX I-IR structures are identified by the arrows. Little of no vimentin staining is associated with these COX I-IR structures. Calibration bars 50 μm in A and 30 μm in B and C.

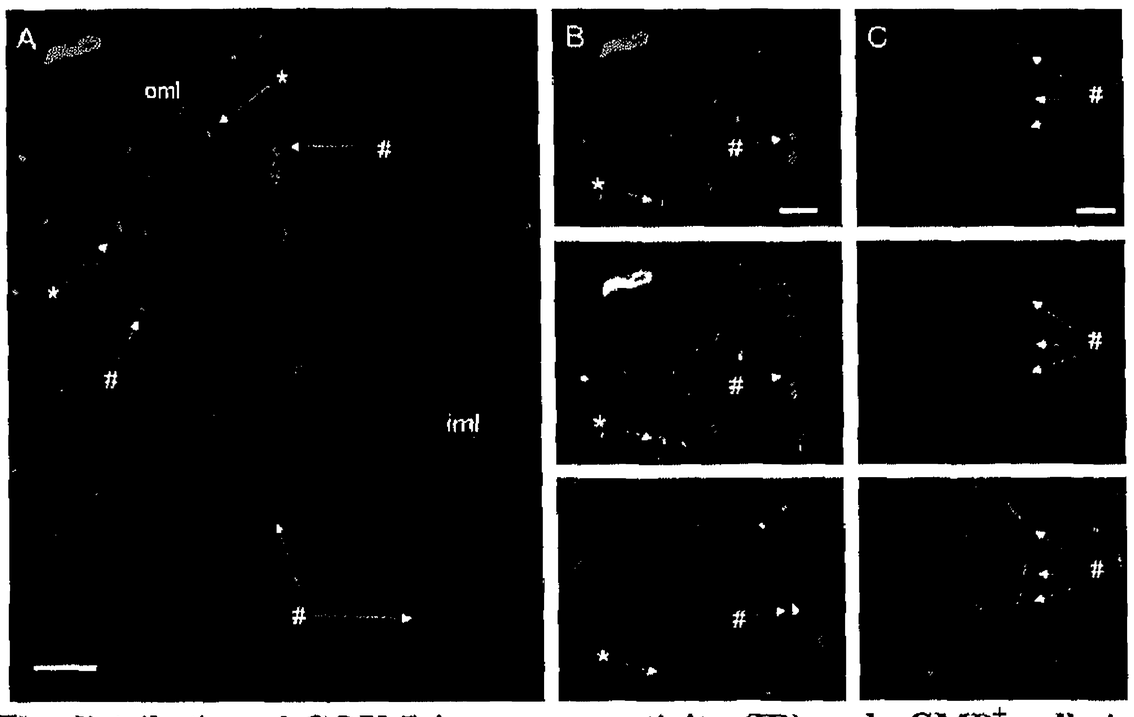


Figure 9. The distribution of COX I immunoreactivity (IR) and cGMP$^+$ cells in the outer muscle layers. A shows an image double labelled with antibodies to cGMP (green) and COX I (red). cGMP$^+$ cells are seen in the outer muscle layer (oml) while there are no cells associated with the inner muscle layer (iml). cGMP$^+$ cells (*) and COX I-IR cells (#) are identified. B and C show regions of the image in A of the outer and inner layers respectively at higher magnification, showing the combined colour image (upper panel: cGMP-green, COX I-red), the cGMP image alone (middle panel) and COX I image alone (lower panel). In B cGMP$^+$ cells are clearly seen (*) while COX I-IR cells are scarce (#). The COX I-IR cells do not show any cGMP staining. C shows the network of COX I-IR cells in the iml and the absence of cGMP staining. Calibration bars 120 μm in A and 40 μm in B and C.

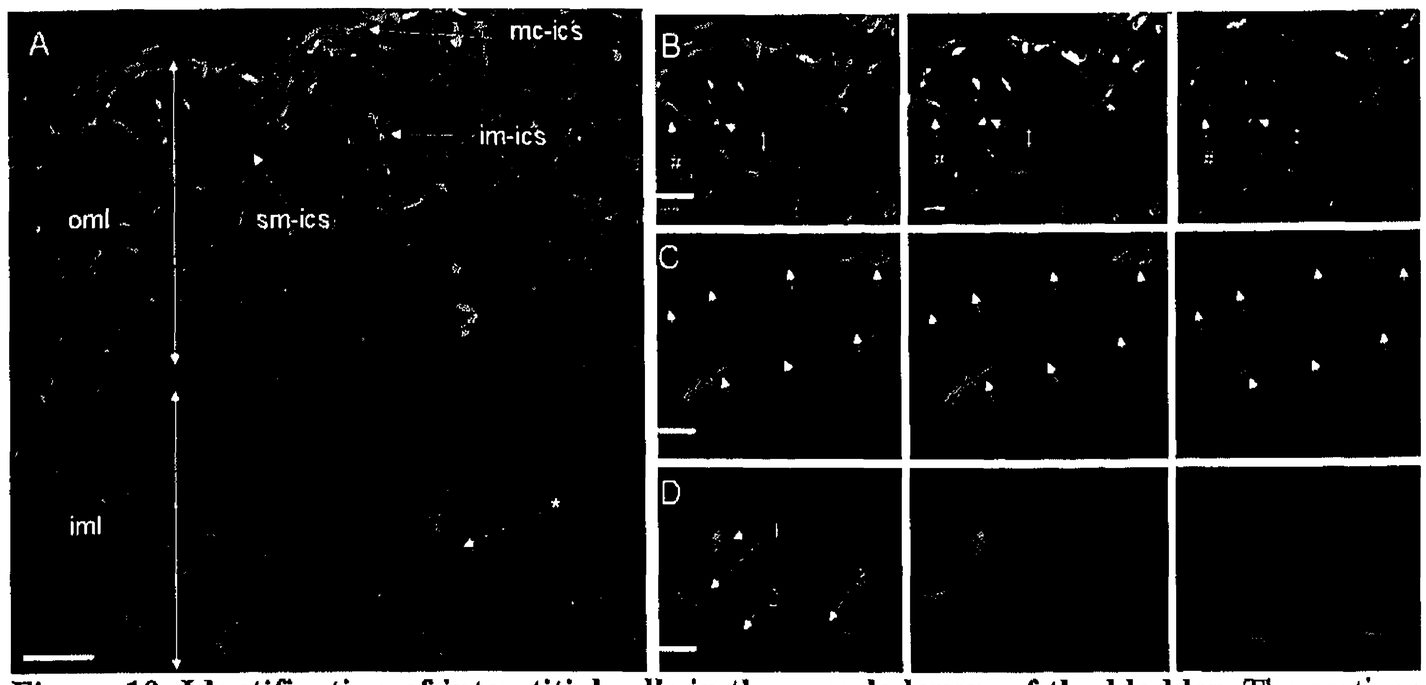


Figure 10. Identification of interstitial cells in the muscle layers of the bladder. The sections are double labelled with antibodies to cGMP (green) and vimentin (red). A shows a low power image identifying the outer (oml) and inner muscle layers (iml). The network of $cGMP^+$ ICs associated with the outer layer and the network of $vimentin^+$ cells in the inner (*) and outer layers are readily seen. The different types if $cGMP^+$ ICs are indicated by the arrows: muscle coat interstitial cells (MC-ICs), surface muscle interstitial cells (SM-ICs) and intra- muscular interstitial cells (IM-ICs). B, C and D illustrate regions of the image in A at higher magnification and showing combined colour image (left panel: cGMP-green, vimentin-red), the cGMP image alone (middle panel) and vimentin image alone (left panel). B shows primarily the SM-ICs. All $cGMP^+$ structure are also $vimentin^+$. C and D focus on the IM-ICs. $cGMP^+$ cells are seen to extend long fine processes. The cell bodies and initial parts of the cell processes are also vimentin positive. D illustrates that there is a variation on the intensity of cGMP and vimentin staining. All IM-ICs express vimentin. However, there are $vimentin^+$ cells which are strongly $cGMP^+$ (1) while others have little cGMP immunostaining (2 and 3). Calibration bars, 100 μm in A, 40 μm in B and 20 μm in C and D.

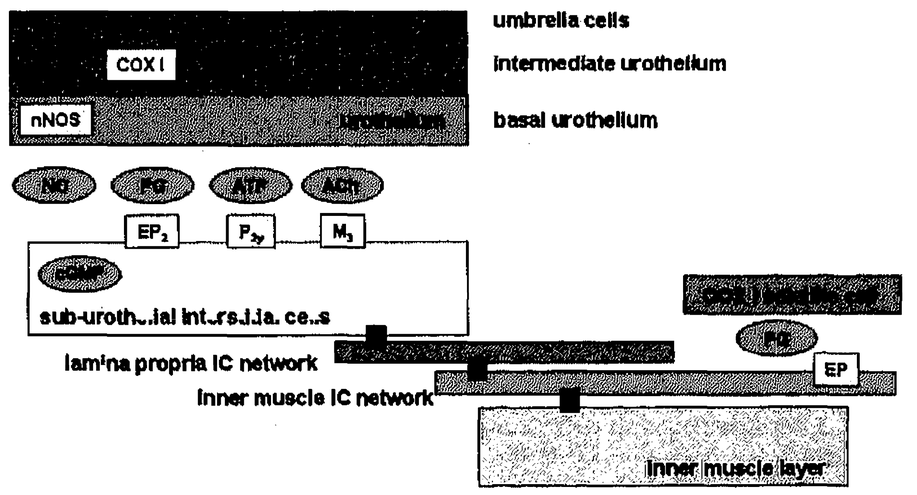


Figure 11. Summary diagram illustrating the arrangement of cells found in the wall of the guinea pig bladder. The urothelium releases NO, PG, ATP and acetylcholine in response to stretch. The production of NO and PG in the urothelium may be interrelated. The substances released may act on afferent nerves but may also act upon the sub-urothelial interstitial cells. This network is in direct contact with a network of vimentin positive interstitial cells which run in the sub-urothelial space of the lamina propria and extend over the surface of the inner muscle bundles. The mechanisms connecting cells in this network is not known but may be via gap junctions (black squares). COX I-IR cells (satellite cells) are associated with the $vimentin^+$ interstitial cell network where they may influence the activity of the network.

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Chapter 6

M_3 muscarinic receptor expression on sub-urothelial interstitial cells

S Grol, PBM Essers, GA van Koeveringe, P Martinez-Martinez, J de Vente and JI Gillespie

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Abstract

Aims The aim of this study was to identify the cells expressing the M_3 muscarinic receptor sub-type in the lamina propria of the bladder.

Materials and Methods The bladders from 5 normal guinea pigs were isolated and fixed in 4% paraformaldehyde. Tissues sections (10 μm) were then cut and stained with antibodies to the type 3 muscarinic receptor (M_3), the interstitial cell marker vimentin and the non specific nerve marker PGP 9.5. The specificity of the antibody to the M_3 receptor was established using the complementary blocking peptide and western blot analysis of HEK cells transfected to express the M_3 receptor protein.

Results The M_3 antibody pre-incubated with its blocking peptide did not show any immunohistochemical staining. Investigating this antibody using HEK cells transfected to express the M_3 receptor protein and control HEK cells showed a single band in the transfected cells and no band in the control cells. M_3 receptor immuno-reactivity (M_3-IR) was detected primarily on a dense network of vimentin positive (vim^+) cells lying immediately below the urothelium: the sub-urothelial interstitial cells (SU-ICs). The M_3-IR was punctuate and appeared to be located on the cell surface. The diffuse cell network of cells in the remaining regions of the lamina propria did not show M_3-IR. Few nerve fibres were associated with the M_3-IR SU-ICs. The M_3-IR SU-ICs were most numerous and prominent in the lateral wall. The number of M_3-IR/vim^+ cells diminished towards the bladder base and were absent in the bladder urethral junction. In the base and urethral junction vim^+ cells were observed which were not M_3-IR. A population of umbrella cells in the lateral wall also showed weak punctuate M_3-IR.

Conclusions Using a well characterised M_3 antibody these observations demonstrate for the first time that the type 3 muscarinic receptor in the lamina propria is located specifically on the sub-urothelial interstitial cells. The physiological role of a these cells is not known and consequently the significance of what appears to be a cholinergic signalling system is unclear. Previously published data has shown that these cells respond to nitric oxide and atrial naturetic peptide with a rise in cGMP and possibly prostaglandin. All of these observations, taken together, suggest that the SU-ICs receive multiple inputs and that they must be part of a complex signalling system in this region of the bladder wall.

Introduction

The importance of cholinergic muscarinic receptors during bladder stimulation was recognised over 100 years ago [1]. It has long been recognised that the M_3 receptors are responsible for activating the detrusor [2]. This has been elegantly demonstrated using M_3 knockout mice where cholinergic activation of the bladder is drastically affected although the animals still appear to void normally [3, 4].

One of the major clinical conditions associated with bladder dysfunction is a sensation of urgency during filling and an increased frequency of voiding. It was originally thought that these symptoms were associated with bladder contractions during the filling phase. Given the importance of M_3 receptors in the activation of bladder contractions M_3 specific anticholinergic drugs were developed to treat the urological symptoms. Clinically, these drugs are effective in reducing symptoms of urgency and frequency. However, it has become apparent that, at therapeutic doses, these drugs do not affect either the non-voiding contractions or the voiding contractions [5-7]. Thus, these drugs must also act on other systems in the bladder. This has led to the suggestion that the anticholinergic drugs act upon sensory mechanisms operating during the filling phase. The search is now on to find these mechanisms. Current thinking has suggested roles for cholinergic mechanisms associated with the urothelium, afferent nerves and the motor/sensory system [8-10].

Molecular studies have shown that the all 5 muscarinic receptors are expressed in the mouse [11] and human bladder [12, 13]. However, it is generally accepted that only M_2 and M_3receptors are involved in the bladder smooth muscle contraction [14]. The relative amounts of M_2 or M_3 receptors in the muscle and urothelial layers of several species, like rat, mouse, and the human, are the not the same [11, 12, 15-18], and the M_2 receptor being present in greater amounts than the M_3 receptor [16, 19]. In order to unravel the different cholinergic elements in the normal and pathological bladder it is essential to know where the muscarinic receptors are located. The most obvious approach would be to use immunohistochemistry. However, the absence of specific and reliable antibodies has limited this way forward. Recent studies on the location of M_2 and M_3 receptors on the human bladder demonstrated their presence either only on smooth muscle cells [20] or on both interstitial cells in the lamina propria and muscle [21]. Another study demonstrated a weak immunostaining of the human urothelium for the M_2 receptor and a strong M_3 immunostaining in this tissue, whereas the real time PCR showed no difference in expression [12]. Unfortunately, in these studies the specificities of the antibodies used was not thoroughly investigated. The aim of the present study was to investigate the localization of the M_3 receptor in the normal guinea pig bladder and in the bladder of animals with a previous inflicted obstruction. A main concern of this study was the characterization of a number of M_3 antibodies, all marketed as specific reagents for this receptor subtype. Although it has been argued that the specificity of antibodies can never be proved [22, 23], we have performed

several tests on these antibodies. We transfected HEK cells with a rodent M_3 receptor as a model system. In addition, we studied all antibodies by Western blotting and, where possible, with preaborbtion experiments. The antibody which passed all these tests were applied to sections of the guinea pig urinary bladder in order to localize the M_3 receptor in this tissue.

Materials and Methods

Guinea pigs (5 male, weight range 270-300g) were killed by cervical dislocation. All procedures were carried out in accordance with guidelines of the University of Maastricht and were in line with the EC guidelines.

Urinary bladder including the proximal urethra was removed from each animal and placed in ice-cold Kreb's solution: NaCl mM, 121.1 mM; KCl 1.87 mM; $CaCl_2$ 1.2 mM; $MgSO_4$ 1.15 mM; $NaHCO_3$ 25 mM; KH_2PO_4 1.17 mM; glucose 11.0 mM and bubbled with 5% CO_2 and 95% O_2 (pH 7.4). Each bladder was divided in two into pieces, a ventral piece and a dorsal piece, and maintained in Kreb's solution containing 1 mM of the non-specific phosphodiesterase inhibitor isobutyl-methyl-xanthine (IBMX: Sigma-Aldrich) at 36°C for 30 minutes. Incubations were terminated by immersing bladder pieces in ice-cold fixative solution of 4% freshly prepared depolymerised paraformaldehyde for 120 min at 4°C. Then, tissues were fixed overnight at 4 °C in 0.1 M phosphate buffer with 10% sucrose and the next day the tissues were placed in at 0.1 M phosphate buffer with 20% sucrose at 4 °C and then washed overnight at 4 °C in 0.1 M phosphate buffer with 30% sucrose. The tissues were placed in Tissue-Tek O.C.T. compound to form a single block. This was then snap-frozen in isopentane cooled in liquid nitrogen. Cryostat sections (10 µm) were cut, such that each section was perpendicular to the urothelial surface. Sections were then thawed on to chrome-alumn-gelatin-coated slides and processed for immunocytochemistry.

Immunohistochemistry

Sections were dried for 60 min at RT followed by three washes with Tris-buffered saline (TBS; pH 7.6), and thereafter incubated overnight with primary antibodies at 4 °C. To visualize PGP9.5 we used rabbit anti-PGP9.5 (1: 2000; Abd Serotec); the selectivity and an estimate of the detection limit of these antibodies have been described previously [24-26]. The mouse antibody against vimentin (Sigma-Aldrich) was diluted 1:5000. The goat antibody against the M_3 receptor (Santa Cruz) was diluted 1:300. Pre-absorption of the anti-M_3 antibody (1:300) was done by incubating the antibody with or without 10 µg/ml of the peptide against which the antibody was raised. Thereafter the antibody solution or antibody plus peptide solution was applied to the sections.

After overnight incubation with the primary antibodies diluted in TBS containing 0.3% (v/v) Triton X-100 (TBS-T), sections were washed in TBS, TBS-T and TBS; each wash step lasted 15 min. Rabbit primary antibody was visualized using Alexa Fluor 488 donkey anti-rabbit IgG (H+L) conjugate

(Molecular Probes), diluted 1 : 100 in TBS-T. Mouse primary antibodies were visualized with Alexa Fluor 488 donkey anti-mouse IgG conjugate (Molecular Probes), diluted 1:100. Goat primary antibodies were visualized with Alexa Fluor 594 donkey anti-goat IgG conjugate (Molecular Probes). Sections were incubated with the secondary antibodies for 60 min at room temperature in the dark. After washing with TBS-T, and TBS, sections were mounted with TBS-glycerol.

In total 5 bladders were used in the study. Typically, for each bladder and for each antibody combination staining was done in duplicate and repeated on at least 2 separate days. Observations were accumulated from the different slides and from the different bladders.

Sections were analysed and photographed using an Olympus AX70 microscope using a x4, x10, x20 and x40 objective. For the detection of Alexa 488 fluorescence we used a narrow band-pass MNIBA-filter and for the detection of Alexa 594 we used a filter with a narrow excitation band, the U-M41007A filter (both filters are from Chroma Technologies). The microscope was equipped with a cooled CCD Olympus Digital video camera F-view. Images were stored digitally as 16 bits images by using the computer program analySIS® Vers.3.0. (Soft Imaging System, Münster, Germany). The number of grey values was reduced using a linear function into 4095. Images were arranged with the program Adobe Photoshop 5.5 or 7.0.1 (San Jose, CA, USA).

Western blots of transfected M_3 cells

HEK-293 cells were grown with Dulbecco's modified Eagle's medium, supplemented with 2 mM L-glutamine, 10% (v/v) fetal bovine serum and penicillin (100 U/ml)/streptomycin sulfate (0.1 mg/ml), at 37 °C in a humidified 5% CO2 environment. Transfections were performed for 15h using ProFection Mammalian Transfection System-Calcium Phosphate (Promega, USA), following manufacturer's recommendations with the ORF Clone of Homo sapiens cholinergic receptor, muscarinic 3 (CHRM3, OriGene, USA).

To obtain cell extracts, growing cultures were rinsed with ice-cold phosphate buffered-saline (PBS) and homogenized on ice bed with 25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 10 µg/ml leupeptin. Mixtures were cleared by centrifugation at 500 x g for 10 min, protein concentration determined and stored at -70 °C. Protein measurements were done using Bovine Albumin Serum dilutions (4 mg/ml, 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml 0 mg/ml) with the Biorad protein measurement device (Bio-Rad Laboratories, Inc., Hertfordshire, CA, USA) according to the manufacturer's instructions.

Western blot analysis were performed under reducing conditions following standard procedures and using the Odyssey infrafed imaging system (Li-cor Biosciences, USA). Primary antibodies used for western blotting were: goat anti- M_3 antibody (Santa-Cruz, SC7474) used in a dilution of 1:500, and mouse anti-rabbit GAPDH (RDI, 5G4-6C5) used in a dilution of 1: 3.000.000. 13063).

Secondary antibodies used were: Donkey anti-Goat IRDey 800 (Odyssey, 926-32214), and Donkey anti-mouse IRDye 700 (Rockland, 610-730-124) both in a dilution of 1:10.000.

Results

Characterisation of the M_3 antibody

In order to find an M_3 antibody suitable for immunohistochemical studies we studied several M_3 antibodies. Figure 1 illustrates the characterization of an antibody (Ab) to the muscarinic type 3 receptor used in this study (Santa Cruz-goat Ab). Panel A shows a section of guinea pig bladder stained with this antibody. The image shows that a strong immuno-fluorescence signal is restricted to cells lying immediately below the urothelium and that there is faint staining in some of the umbrella cells. Staining was also seen associated with the smooth muscle (not shown). One approach to assess whether this staining is specific is to interfere with the reactive site of the antibody by pre-incubation with the peptide to which it was raised. Panel B shows that all of the staining is removed with the pre-absorbed antibody. This is one indication that the Ab binding is specific for the epitope to which the Ab was raised. A second approach was to demonstrate that the antibody reacts specifically with the M_3 receptor by Western blot studies. This was done using Western blot examination of homogenates from cells which do not express the M_3 receptor (control cells) and from cells which were transfected with DNA encoding for the M_3 receptor (transfected cells, see above). Panel C shows a western blot demonstrating that the antibody detects a protein band of approximately 100 kD, the correct molecular weight for the M_3 receptor. This band is only found in the transfected cells. Therefore, based on these criteria we have an antibody which can be reliably used to explore the distribution of M_3 receptors in the guinea pig bladder

Location of the M_3 antibody

Muscarinic type 3 receptors were found to be located on the dense population of sub-urothelial interstitial cells (SU-ICs) which lie immediately below the urothelium (Figure 2). These cells express vimentin, identifying them as of mesenchymal origin. The diffuse network of vimentin positive cells which lie within the bulk of the lamina propria (lamina propria interstitial cells (LP-ICs) did not express M_3 receptors indicating at least two distinct types of interstitial cell in the lamina propria. The staining associated with the SU-ICs appeared to be concentrated in the cell bodies and is particularly intense in the region of the nucleus. However, staining was also clearly associated with cell processes (Figure 2B and C). It is noteworthy that the staining appears to be punctuate with intense aggregations of fluorescence appearing through out the cells. Figure 1 A shows that there are a small number of the umbrella cells which stain weekly with the M_3 antibody (+). This is again illustrated in Figure 3. Here also there appears to be a punctuate distribution of M_3 receptors. Not all

umbrella cells stain with the M_3 antibody indicating a possible heterogeneity within these epithelial cells.

Regional differences in distribution of the M_3 antibody

An interesting observation was that the interstitial cells expressing the M_3 receptors were more prominent in the lateral wall compared to the bladder base and in the urethra (Figure 4). In the lateral wall the SU-ICs layer could be up to 5-8 cells deep with the vast majority of the cells expressing the M_3 receptor. Towards the bladder base this layer thinned and was only 1-3 cells deep and was completely absent in the region of the bladder urethral junction (Figure 4 B and A respectively). Comparing panels B and C it is also apparent that the number of clusters of M_3 receptors appears to be higher in the cells of the lateral wall compared tot the base. This differential expression might suggest a localized regulation of receptor expression. Close examination of these different regions revealed the presence of SU-ICs (vimentin positive) that did not express M_3 receptors (M_3 negative). Few such cells were seen in the lateral wall while they were more abundant at the base (Figure 5). In the bladder urethral junction vimentin positive cells were found in a sub-urothelial layer but these cells did not demonstrate M_3 receptors.

It was also noted that the M_3 positive SU-ICs were not directly associated with nerve fibres. Nerves were seen to run within the M_3 positive SU-ICs layer but the density was low. However, in the region of the bladder/urethral junction where there are numerous nerve fibres the M_3 positive SU-ICs were absent.

Discussion

In the present study we used antibodies which detect the M_3 type muscarinic receptors in the guinea pig urinary bladder wall. It can never be ruled out completely that the antibody exhibits some non-specific binding [22, 23] and also that it might also recognise other muscarinic receptor iso-forms. By transfecting HEK cells with the M_3 receptor and the demonstration that this receptor is actually present using the two antibodies, in combination with the demonstration of M_3 receptor reactivity in Western blots of whole guinea pig bladder homogenates, and in addition the complete absence of immunostaining in the tissue after preabsorbtion of the antibodies with the respective peptides, we have met the criteria necessary to characterise polyclonal antibodies. However, as antibodies are affinity reagents there will always be room for discussion, as there are no pharmacological/physiological model systems available to test the M_3 antibodies as e.g. have been used characterizing the cGMP antibody [27]. However, there is a concordance between our immunohistochemical results and the physiological data already available and presented during the introduction of this paper lending additional support to our viewpoint that using these antibodies it is indeed possible to visualize the M_3 receptor. Therefore, this discussion is presented on the basis that the antibody identifies cells expressing muscarinic receptors.

The urothelium has been shown to release PG [28-31], ATP [32], NO [32-34] and ACH [11, 35] primarily in response to stretch. This release is thought to be the initial step involved in a system involved in detecting bladder volume. As the bladder fills the urothelium is stretched and substances are released which results in the activation and modulation of afferent nerves. There is experimental evidence demonstrating the modulation of afferent nerve firing by ATP [36] and indirect evidence for the involvement of NO [37] and ACh [38] in the modulation of the afferent limb of the micturition reflex. PG, ATP, ACh and NO appear to be released through out the bladder, in the lateral wall and dome. In these regions the density of afferent nerves is low. Therefore, is seems likely that substances released in these regions sub-serve functions other than direct neuro-activation or neuro-modulation.

The possibility has been considered that there are more indirect involvement of substances released from the urothelium and afferent nerves [6, 39]. For example, it has been suggested that there are specialized cells in the lamina propria, myofibroblasts, which lie close proximity to afferent nerve fibres [40]. Cells purported to be these myofibroblasts respond to ATP [41]. This has led to the idea that the myofibroblasts contract in response to ATP so distorting and activating the adjacent afferent nerves. Although an interesting concept there is no direct experimental evidence for the operation of such an arrangement in the bladder wall. Based on the original description of myofibroblasts in the lamina propria is seems unlikely that these distributed cells are those described as SU-ICs. It is more likely that the myofibroblasts lie within the regions of LP-ICs. A further aspect, suggesting that myofibroblasts and SU-ICs are different, is that the SU-ICs layer in the lateral wall is poorly innervated with sensory fibres (Figure 6) [42].

The absence of nerves associated with the SU-ICs layer further suggests that these cells do not have an efferent supply and so are not under the influence of any significant neural control. If the M_3 receptors are functional and activated by ACh then a key question is the source of the ACh. It has been shown that ACh is released from the urothelium in response to stretch [43]. Therefore, this is a potential source. The data from these human studies suggests that the outer cells of the urothelium that express the enzyme choline acetyltransferase (ChAT) and are thus responsible for the synthesis of ACh. A similar observation, locating ChAT to the umbrella cells has been made of the guinea pig urothelium (de Vente and Gillespie, unpublished observations). Thus, the likely elements of this signalling system involve the release of ACh from the urothelium and the activation of the underlying sub-urothelial cells.

The network of vimentin positive SU-ICs extends from the bladder base, over the lateral wall and into the dome. The co-expression with the M_3 receptor is primarily located in the lateral wall where the cell layer is several cells thick. This region must therefore represent the region of the bladder where this mechanism is most active and most relevant. Towards the bladder urethral junction this system disappears, the expression of the M_3 receptor declines and

the proportion cells which are only vimentin positive is increased. The M_3 mechanism is therefore absent in the region of the bladder urethral junction.

The cells which lie in the sub-urothelium of the bladder were first described by Smet [44] as a population of cells that responded to exogenous nitric oxide with a rise in cGMP. The physiological role of these cells and the significance of this responsiveness was not considered. Subsequent work has confirmed the sensitivity of this cell layer to NO and shown that they lie in close proximity to cells in the basal urothelium which express neuronal nitric oxide synthase, the enzyme responsible for generating NO [45]. This suggested that there is a transfer of signals between the urothelium and SU-ICs.

It has been shown that, in the guinea pig, the basal and intermediate cells of the urothelium express the cyclo-oxygenase enzyme COX I [46]. Therefore, these cells are likely to be responsible for the generation of prostaglandin from the urothelium. The cellular target for this prostaglandin is not known but there is preliminary data suggesting that the SU-ICs express the type 2 prostaglandin receptor (EP_2) [47]. In other tissues the EP_2 receptor is linked to adenylate-cyclase and, when activated, generates a rise in intracellular cAMP. Thus, a second urothelial derived signal, prostaglandin, has the potential to interact with the SU-ICs and to generate a second cascade of intracellular signals.

The present data shows that these SU-ICs also express type 3 muscarinic receptors suggesting that they can respond to ACh. M_3 receptors belong to the sub-set of muscarinic receptors that couple to G proteins and when activated result in a rise in diacylglycerol and inositol-trisphosphase (IP_3). The IP_3 generated then initiating the release of intracellular calcium. This series of events may occur within the SU-ICs.

It would thus appear that the SU-ICs receive multiple inputs via the urothelial derived signals. These signals are likely to be involved in activating or modulating different pathways within the SU-ICs. These systems may act synergistically to produce or modulate a specific response. Alternatively, they may be antagonistic and, depending upon their level, integrate to produce a specific response. The SU-ICs may therefore be a point of integration of signals derived from the urothelium. The signals generated by the urothelium and the receptors and pathways they may act upon associated with the SU-ICs are illustrated in the cartoon in Figure 7. The output of the SU-ICs and the physiological system that they might control or regulate are not known.

It has been known for many years that the urothelial cell layer of the bladder expresses type 2 and type 3 muscarinic receptors with amount of M_2 being greater than M_3. This conclusion is based upon studies of the relative expression of the appropriate mRNA and from studies of radio-ligand binding [18, 48]. The techniques used cannot identify the specific cell types expressing these receptors and the question of the location of these receptors has remained open for some considerable time. Although the present observations do not yield any information on the location of the M_2 receptors they do point to the SU-ICs as the location of the M_3.

In conclusion, the characterization of an antibody which is specific for the M_3 sub-type of the muscarinic receptor has pointed to a novel signalling system in the bladder wall. The suggestion that the SU-ICs represent a cell type which can integrate the plethora of urothelial signals is an additional novel concept. These ideas now pose intriguing questions regarding the generation and modulation of the urothelial signals, how these signals interact and influence SU-ICs and finally what systems are activated following SU-IC activation. Overall, the major driving question must be to discover the physiological system that these processes underpin.

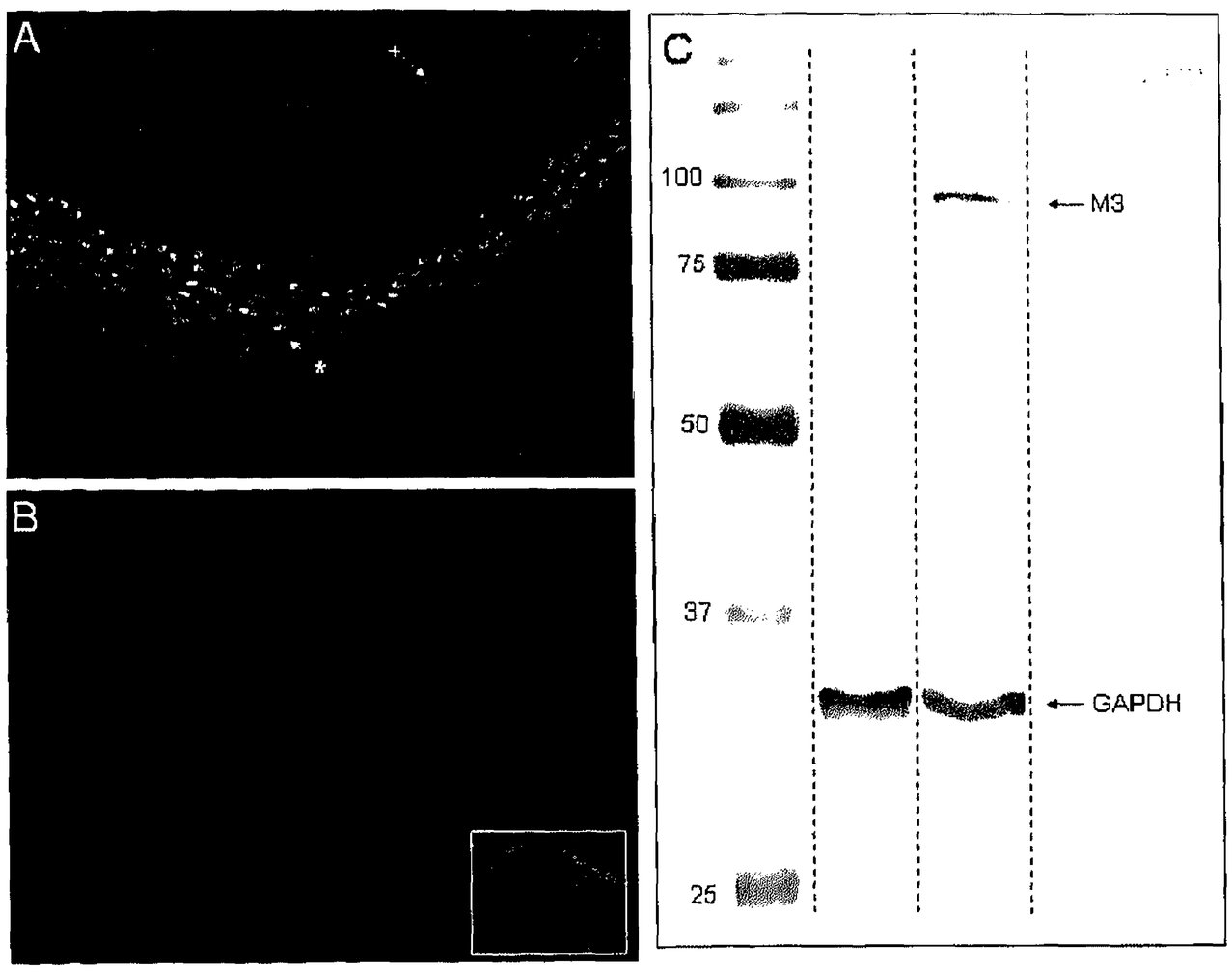


Figure 1. Specificity of the M_3-antibody. Panel A shows M_3-immunoreactivity (M_3-IR) in a layer directly below the urothelium. Panel B shows the same M_3-antibody pre-incubated with its blocking peptide. The M_3-IR in cells directly below the urothelium and in urothelial cells has disappeared. Panel C shows a western blot of HEK cells with and without transfection with the M_3-receptor. The first lane shows the control HEK cells stained with our M_3 antibody and GAPDH. The M_3 antibody does not show any staining in the non-transfected cells (lane 1), while there is a clear band at the height of 95kD in the M_3-transfected cells (lane 2). GAPDH shows the same amount of protein in both lanes.

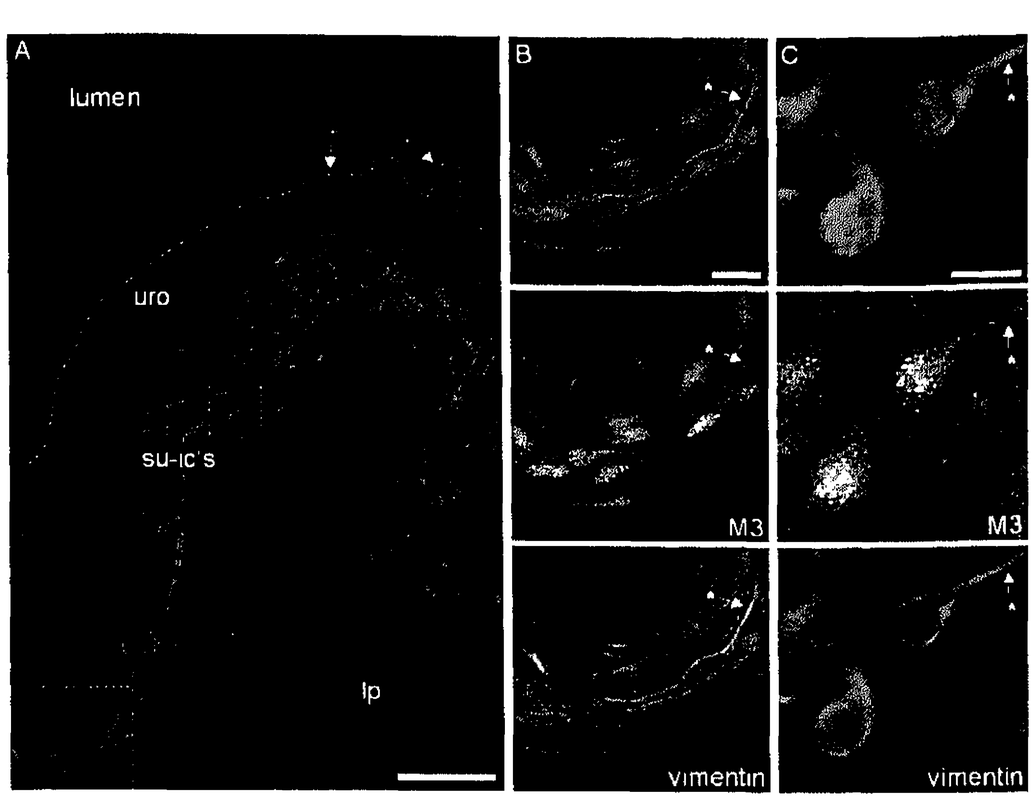


Figure 2. M_3-immunoreactivity (M_3-IR) in sub-urothelial interstitial cells. All panels are stained for vimentin (vim, green) and M_3 (red). Panel A shows a low power image of the urothelium (uro), sub-urothelium and lamina propria (lp). The sub-urothelial interstitial cells (SU-ICs) show immuno-reactivity for both vim and M_3. Panel B and C show in more detail the SU-ICs. The M_3-IR on these cells is punctuated and located on both the cellbody and their processes (*). Calibration bars 50μm in A, 20μm in B and 10μm in C.

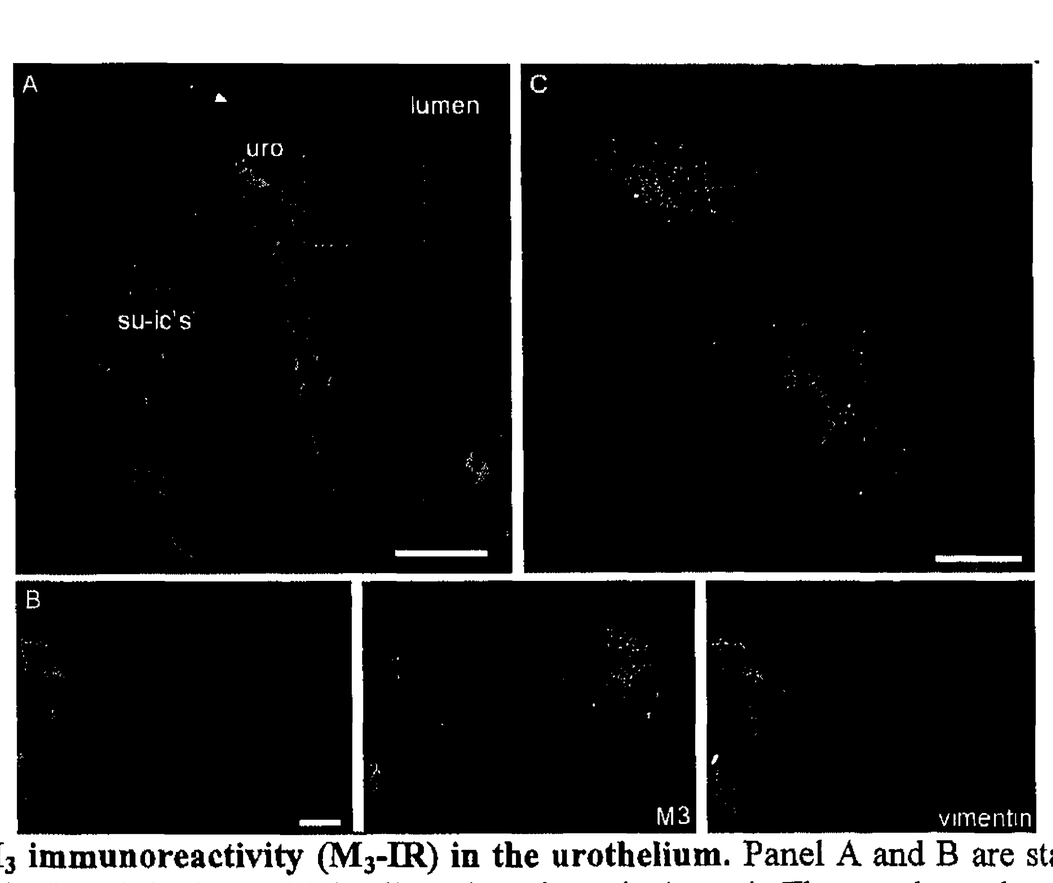


Figure 3. M_3 immunoreactivity (M_3-IR) in the urothelium. Panel A and B are stained for the M_3 receptor (red) and the interstitial cell marker vimentin (green). The panels on the right of panel B show the original pictures of which panel B is constructed. Panel C is stained for the M_3 receptor. Panel A illustrates the M_3-IR in the sub-urothelial interstitial cells (SU-ICs) and in the umbrella cells (*). Panel B shows at high magnification a detail of panel A. The M_3-IR appears to be punctuate in both the umbrella cells and the SU-ICs. Panel C shows in further detail the punctuate M_3-IR in the umbrella cells of a different bladder. Calibration bars 50 μm in A and 10 μm in B and C.

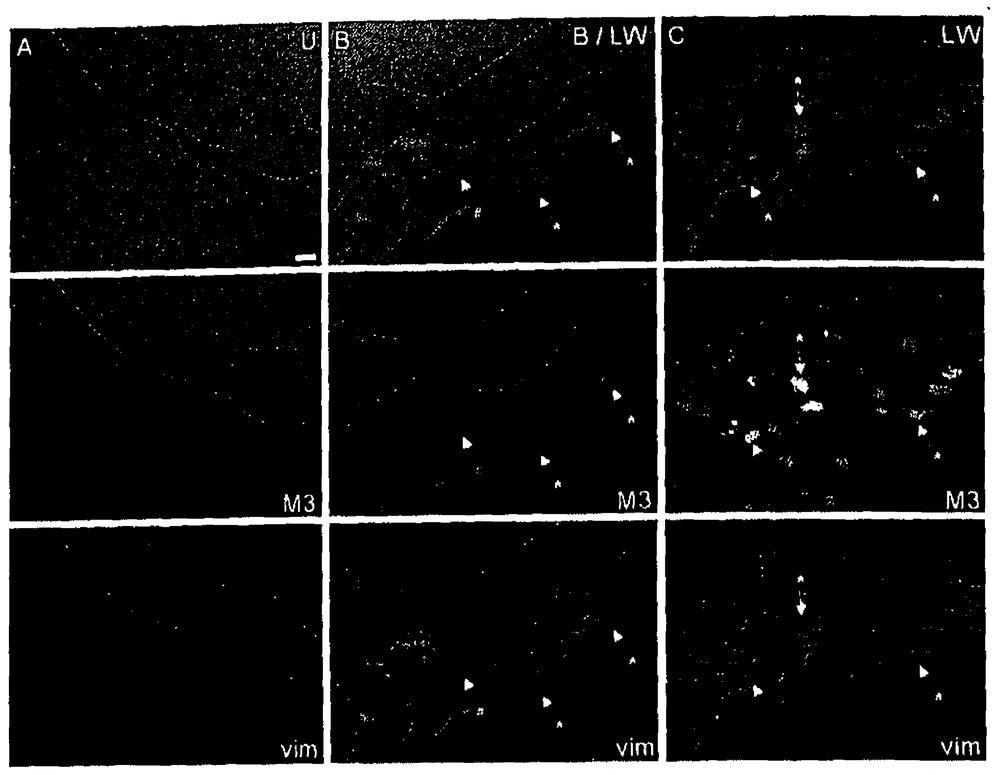


Figure 4. Distribution of M_3-immunoreactivity (M_3-IR) on sub-urothelial interstitial cells (SU-ICs) in different regions of the bladder. Panel A, B and C are all stained for the M_3 receptor (red) and the interstitial cell marker vimentin (green). The panels below A, B and C show the original pictures of which panels A, B and C are constructed. Panel A shows a section of the urethra (U). No SU-ICs were located in this area. Panel B shows a section of the transition zone of the base/lateral wall (B/LW). Here there are SU-ICs visible. There appear to be two subsets of SU-ICs, vim+ / M_3- SU-ICs (#) and vim+ / M_3+ SU-ICs (*). In the lateral wall (panel C, LW) the SU-IC layer appears to be thicker and here almost all SU-ICs show M_3-IR (*). Calibration bar in A is for A, B and C; size 10 μm.

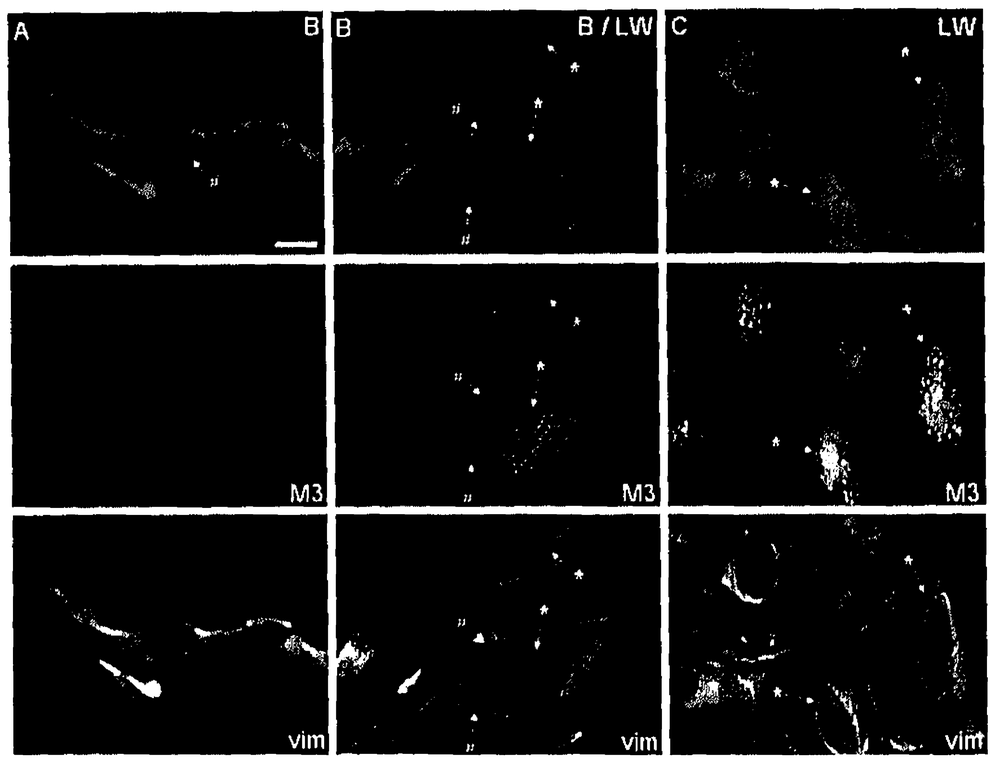


Figure 5. Differences in M_3-immunoreactivity (M_3-IR) in sub-urothelial interstitial cells (SU-ICs). Panel A, B and C are all stained for the M_3 receptor (red) and the interstitial cell marker vimentin (green). The panels below show the original images of panel A, B and C. Panel A shows a SU-ICs (#) of the base (B). This cell shows no M_3-IR. Panel B shows SU-ICs of the transition zone base/lateral wall (B/LW). In this area there appears to be two types of interstitial cells; vim+/ M_3+ cells (*) and vim+/ M_3- cells (#), while in the lateral wall (panel C, LW) there is only one type of su-ics: vim+/M_3+ su-ics (*). Calibration bar in A is for A, B and C; size 10 μm.

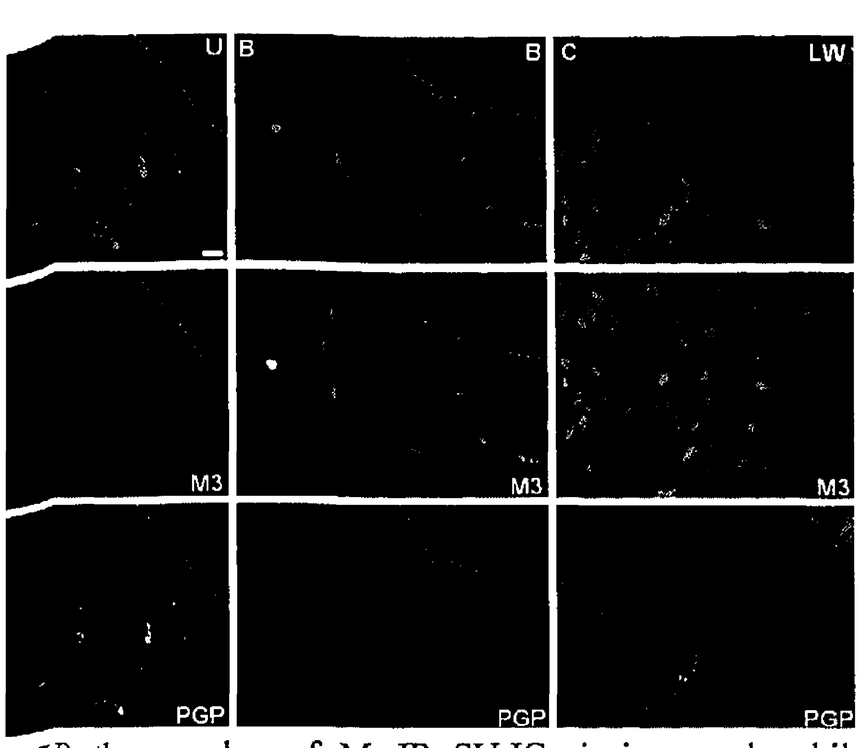
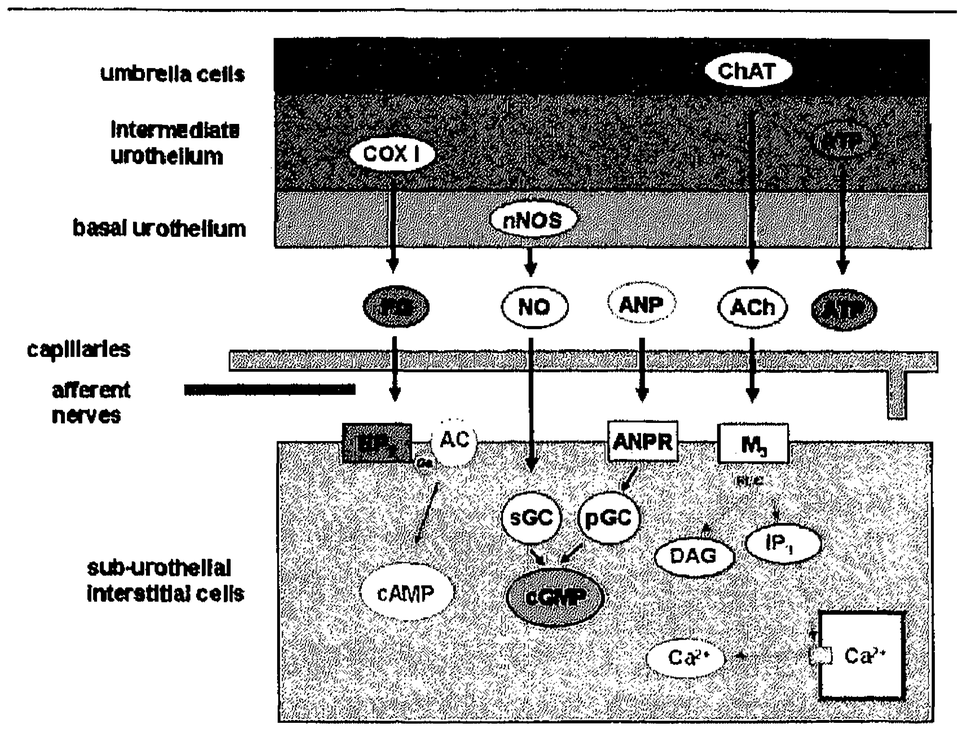


Figure 6. M_3-immunoreact ve (M_3-IR) sub-urothelial interstitial cells (SU-ICs) in relation to the distribution of nerves. Panel A, B and C are all stained for the M_3 receptor (M_3, red) and the non-specific neuronal marker PGP9.5 (PGP, green). The panels below show the original images of panel A, B and C. In the urethra (U) there are no M_3-IR SU-ICs, while there is a dense network of nerves (panel A). In the base (B) there are M_3-IR SU-ICs, but only a sparse distribution of nerves (panel B). In the lateral W) the number of M_3-IR SU-ICs is increased, while there is no difference in the tion of nerves. Thus, it can be concluded that there is no relation between the distribution es and the distribution of M_3-IR SU-ICs. Calibration bar in A is for A, B and C; size 10



7. Cartoon illustrating the output of urothelial signals and possible interactions of ignals on the sub-urothelial interstitial cells in the guinea pig bladder. The output of othelium including prostaglandin (PG), nitric oxide (NO), acetylcholine (ACh) and ine trisphosphate (ATP) are all well documented [11, 28-35]. The present data demonstrate sence of M_3 receptors on the SU-ICs. EP_2 receptors have been demonstrated immuno-emically and atrial naturetic peptide (ANP) receptors inferred from the actions of ous ANP [47, 49]. NO and ANP induce a rise in cGMP. Based on published data activation generates a rise in cAMP via adenylate cyclise (AC) and activation of M_3 causes a rise in llular Ca^{2+} from intracellular stores and diacylglycerol (DAG). The physiological system inned by these signalling elements on the SU-ICs has yet to be identified.

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Chapter 7

M_3 muscarinic receptor like immuno-reactivity (M_3-IR) in the sham-operated and obstructed guinea pig bladder

S Grol, R de Jongh, GA van Koeveringe, PEV van Kerrebroeck, M Markerink-van Ittersum, J de Vente and JI Gillespie

Abstract

Type M_3 muscarinic receptors are central to bladder function. There is growing awareness that M_3 receptors are located not only on the smooth muscle but also associated with other cell types and mechanisms. The aim of this study was to identify the cellular location of M_3 receptors in bladders from normal guinea pigs and guinea pigs with surgically induced bladder neck damage. In the sham-operated bladder M_3 like immuno-reactivity (M_3-IR) was associated with nerves: a sub-population of nerves in the lamina propria, a sub-population of nerves within the muscle bundles and the intra-mural ganglia. M_3-IR was also associated with vimentin positive interstitial cells. Different types of cell were identified: sub-urothelial cells (SU-ICs), cells in the lamina propria (LP-ICs) and cells associated with the muscle either on the surface (SM-ICs) or within the bundles (IM-ICs). In bladders with previous damage to the bladder neck there was a hypertrophy of the wall accompanied by a loss of intramural nerves and ganglia. The SU-ICs appeared little changed but LP-ICs were more abundant, the cells having long bifurcating processes. There was an increase in SM-ICs and IM-ICs. Between the muscle bundles the cell bodies of these cells appeared in clusters forming 'nodes'. Thus, there is a major remodelling of the bladder wall following damage to the bladder neck. The loss of nerve and the up-regulation of interstitial cells expressing M_3-IR may underlie major functional changes. The hypothesis is put forward that the augmentation of the interstitial cell network modifies the non-voiding phasic activity and increases motor/sensory output resulting in sensations of urgency. Furthermore, it is argued that the cholinergic receptors associated with this interstitial cell network are a therapeutic target for anticholinergic drugs.

Introduction

The importance of cholinergic muscarinic receptors during bladder stimulation was recognised over 100 years ago (Langley 1895). We now know that, based on analyses of total mRNA, all five receptor subtypes (M_1, M_2, M_3, M_4 and M_5) are found in both in the urothelial and muscle layers (Siglia *et al.* 2002; Tyagi *et al.* 2006; Ruggieri and Braverman 2006). Ligand binding receptor assays suggest that the most abundant forms are M_2 and M_3 with M_2 being present in greater amounts than M_3 (Mansfield *et al.* 2005; Ruggieri and Braverman 2006). It has long been recognised that the M_3 receptors are responsible for activating the detrusor (Chess-Williams 2002). This has been elegantly demonstrated using M_3 knockout mice where cholinergic activation of the bladder is drastically affected although the animals still appear to void normally (Matsui *et al.* 2000; Matsui *et al.* 2002). At present the precise role of the M_2 receptor sub-type in the bladder is controversial.

One of the major clinical conditions associated with bladder dysfunction is a sensation of urgency during filling and an increased frequency of voiding. It was originally thought that these symptoms were associated with bladder contractions during the filling phase. Given the importance of M_3 receptors in the activation of bladder contractions M_3 specific anticholinergic drugs were developed to treat the urological symptoms. Clinically, these drugs are effective in reducing symptoms of urgency and frequency. However, it has become apparent that, at therapeutic doses, these drugs do not affect either the non-voiding contractions or the voiding contraction (Andersson and Yoshida 2003; Andersson and Arner 2004; Finney *et al.* 2006). Thus, these drugs must also act on other systems in the bladder. This has led to the suggestion that the anticholinergic drugs act upon sensory mechanisms operating during the filling phase. The search is now on to find these mechanisms. Current thinking has suggested roles for cholinergic mechanisms associated with the urothelium, afferent nerves and the motor/sensory system (see de Groat 2004; Fry *et al.* 2004; Gillespie 2005).

In order to unravel the different cholinergic elements in the normal and pathological bladder it is essential to know where the muscarinic receptors are located. Remarkably, there are few published studies which have succeeded in addressing this question. Molecular studies have suggested differences in the relative amounts of M_3 and M_2 receptors in the muscle and urothelial layers (Ruggieri and Baverman 2006) and also in different regions of the bladder (Sigala *et al.* 2002). Such approaches do not allow the location of receptors to specific cell types. As a result functional interpretations of these molecular approaches have focused on the major cell types in the bladder: the urothelium and muscle. However, other cell types which may be in low abundance may have cholinergic inputs that may be central to bladder function.

The most obvious approach would be to use immunohistochemistry. However, the absence of specific and reliable antibodies has limited this way forward. One recent study on human bladder located M_2 and M_3 receptors to interstitial

cells in the lamina propria and muscle (Mukerji *et al.* 2006). Unfortunately, this work did not demonstrate the specificity of the antibodies and the observations must be regarded with caution. In this study we present immunohistochemical data using an antibody which appears to be specific for the M_3 receptor based on its molecular characterisation using western blot analysis (Santa Cruz laboratories (Ab SC-7474); (Espanol and Sales 2004; Rimmaudo *et al.* 2005) and the use of its blocking peptide. However, we can never be absolutely certain of the specificity of this or any other antibody. Hence we describe the staining and M_3 immuno-reactivity (M_3-IR). With this caution the M_3-IR appears to be located on different cell types in the bladder wall: muscle, interstitial cells, ganglia and nerves. Also, there appear to be differences in the pattern of M_3-IR in bladders of sham operated animals and animals with previous surgical damage to the bladder neck. These observations are discussed in terms of normal bladder function and in association with the changes seen in the pathological bladder.

Materials and Methods

The studies were performed in 14 male guinea pigs (Hartley strain) weighing between 266-299 grams. The guinea pigs were housed in a temperature-and light - (12h light/dark cycle) controlled room and allowed free access to food and water. The institutional animal care and use committee of Maastricht University approved all the animal experiments conducted in this study. All procedures were in line with the NIH guidelines.

Surgical procedure: The procedures used were similar to those reported previously (Mostwin *et al.* 1991). Briefly, in 8 animals a partial outflow obstruction was induced while 6 underwent sham operation. The protocol based on this guinea pig model for gradual urethral obstruction was used as described by (Mostwin *et al.* 1991). Animals were sedated with ketamine (40 mg/kg) and xylazine (3 mg/kg) administered intraperitoneally. Once sedation was achieved and the animals were prepared under sterile conditions, the bladder neck and urethra were exposed via a vertical midline abdominal incision. A silver jeweler's jump ring (1.8 mm internal diameter) was placed loosely around the proximal urethra. Bladder outlet obstruction occurred during subsequent growth of the animal. The analgesic medication flunixinum (5mg/kg s.c.) and the antibiotic gentamicin (5mg/kg i.m.) were administered once postoperatively. Sham surgery was performed in an identical manner, except for inserting the ring around the urethra.

Four weeks after the operation, the weight gain in the sham operated animals was 190 $\pm$ 52 grams compared to the animals with a bladder outflow obstruction 112 $\pm$ 56 grams. The bladder weights of sham operated and obstructed animals were 400 $\pm$ 73 mg and 1568 $\pm$ 500 mg respectively.

The bladders of these animals were used to study NO-mediated cGMP synthesis as described (De Jong *et al.* CTR 330:147-60 2007). The present study was done on sections from these animals. After 4 weeks, the guinea pigs were killed

by cervical dislocation. The urinary bladder was removed from each animal and placed in ice-cold Kreb's solution containing 121.1 mM NaCl, 1.87 mM KCl, 1.2 mM CaCl2, 1.15 mM MgSO4, 25 mM NaHCO3, 1.17 mM KH2PO4, 11.0 mM glucose, bubbled with 5% CO2 and 95% O2 (pH 7.4). The procedures for isolation, stimulation with nitric oxide (NO) and detection of cGMP were as described previously (Gillespie *et al.* 2004). The lateral wall bladder was dissected into sections (approximately 20 mm2), each of which was maintained in 2 ml Krebs' solution containing 1 mM of the non-specific phosphodiesterase inhibitor isobutyl-methyl-xanthine (IBMX: Sigma-Aldrich) at 36°C for 30 min. Tissues were either stimulated with 100 μM of the NO donor diethylamine-NONOate (DEANO; Sigma-Aldrich) or left in control medium containing only IBMX. The NO donor was prepared immediately before use, added directly to the bathing solution and incubated with the tissue for 10 min. Incubations were terminated by immersing bladder pieces in ice-cold fixative solution of 4% freshly prepared depolymerised paraformaldehyde for 120 min at 4°C. Then, tissues were washed at 4 °C in 0.1M phosphate buffer containing 10% sucrose (24 h), 20% sucrose (24h) and 30% sucrose (24h). The tissues from the lateral wall were then snap-frozen with CO_2 in Tissue-Tek O.C.T. compound to form a single block. Cryostat sections (10 μm) were cut, such that each section was perpendicular to the urothelial surface. Sections were then thawed on to chrome-alumn-gelatin-coated slides and processed for immunohistochemistry. Sections were dried for 20 min at RT followed by three washes with Tris-buffered saline (TBS; pH 7.6), and thereafter incubated overnight with primary antibodies at 4 °C.

Primary antibodies were diluted in TBS containing 0.3% (v/v) Triton X-100 (TBS-T), sections were washed in TBS, TBS-T and TBS; each wash step lasted 10 min. The antibodies used here: rabbit anti-PGP-antibody (Biogenesis) diluted 1:1000; goat anti- M_3 antibody (Santa-Cruz, C-20) used in a dilution of 1:300; rabbit anti-ChAT-antibody (Chemicon) used 1:100; mouse anti-vimentin- antibody (Chemicon) used 1:100 and CGRP (Chemicon) used 1:200. The goat primary antibodies were visualized using donkey anti-goat-CY3 conjugate IgG (Jackson) diluted 1:100 in TBS-T. Rabbit primary antibodies were visualized with Alexa Fluor 594 donkey anti-rabbit IgG conjugate (Molecular Probes), diluted 1:100. Sections were incubated with the secondary antibodies for 60 min at RT in the dark. After washing with TBS-T, and TBS, sections were mounted with TBS-glycerol.

Pre-absorption of the anti- M_3 antibody (1:300) was done by incubating the antibody with or without 10 μg/ml of the peptide (SC7474P, Santa Cruz) against which the antibody was raised overnight at 4°C. Thereafter the antibody solution or antibody plus peptide was applied to the sections. Pre-absorption of the antibody with the peptide abolished antibody binding indicative of a high degree of antibody specificity. Information available in the antibody data sheets from Santa Cruz indicates that, using western blot analysis, this M_3 antibody recognises the 75kD M_3 receptor protein (Espanol and Sales 2004; Rimmaudo

et al. 2005) and that this specific protein binding is also removed by pre-incubation of the antibody with the blocking peptide. Thus, it is possible to conclude that this antibody is capable of recognising specifically the type 3 muscarinic receptor. However, there is always uncertainty with respect to such specificity. For this reason we have described the results as determining M_3 receptor immuno-reactivity rather than a categorical statement that we are seeing the M_3 receptors.

Typically, for each bladder and for each antibody combination staining was done in duplicate and repeated on at least 2 separate days. Observations were accumulated from the different slides and from the different bladders.

Sections were analysed and photographed using an Olympus AX70 microscope. For the detection of Alexa 488 fluorescence we used a narrow band-pass MNIBA-filter and for the detection of Alexa 594 we used a filter with a narrow excitation band, the U-M41007A filter (both filters are from Chroma Technologies). The microscope was equipped with a cooled CCD Olympus Digital video camera F-view. Images were stored digitally as 16 bits images by using the computer program Cell^P (Soft Imaging System, Münster, Germany). The number of grey values was reduced using a linear function into 4095. Images were arranged with the program Adobe Photoshop 5.5 or 7.0.1 (San Jose, CA, USA) without further processing unless indicated otherwise.

Results

Antibody specificity and muscarinic receptor type 3 immunoreactivity

Immuno-reactivity to the M_3 antibody is seen in cells in the immediate sub-urothelial cell layer, the sub-urothelial interstitial cell layer (SU-ICs), lamina propria and inner muscle (see figure 1A). Pre-incubation of the antibody with the peptide against which the antibody was raised abolished staining (see figure 1c). Thus, the antibody is able to detect structures with M_3-IR.

Bladders from sham operated control animals

The structures within the lamina propria expressing M_3-IR are shown in Figures 2-4. In Figure 2 panels A and D show low power images of sections double stained for vimentin (green) and M_3-IR (red). Cells in the sub-urothelial layer are densely packed and stain intensely for vimentin. Vimentin positive cells are also found in the lamina propria but here the cells are sparse. The panels B, C, and E show that M_3-IR is located immediately below the urothelium in the sub-urothelial interstitial cells. A comparison of panels B and C shows that the number of M_3-IR cells can vary in different regions of the sub-urothelial layer. Panel E illustrates the sub-urothelial interstitial cells. F shows a region of this image at higher magnification illustrating the observation that the M_3-IR is located in the region of the interstitial cell bodies. However, M_3-IR is also located in the sub-urothelial cell processes although here the intensity of the staining is week (Figure 3).

Nerves, presumably sensory nerves, are found in the sub-urothelial space. Examination of sections double stained for the non-specific neuronal marker PGP 9.5 and the M_3 antibody suggest that there is one population of nerves which express M_3-IR and a second population which does not (Figure 4 A and B). In the muscle layers M_3-IR is associated with the smooth muscle (Figure 4 C) but it can also be located to nerve fibres identifiable by PGP 9.5 co-staining ((+) Figure 4 D and E). Interestingly, as in the lamina propria, there is a sub-population of PGP 9.5 fibres which do demonstrate M_3-IR ((‡) Figure 4 D and E). Thus, there appear to be different types of nerves in both the lamina propria and muscle layers.

Ganglia are found in the sub-urothelial layer where they form an interconnecting network. Previous studies have shown that the cell bodies of the neurones forming these ganglia receive inputs from two sets of fibres: fibres that stain positively for the enzyme synthesising acetylcholine, choline acetyl-transferase (ChAT) and fibres containing calcitonin gene related peptide (CGRP) (See Figure 5 A, B and C). Figure 5 D shows that M_3-IR can be detected on the ganglionic nerve cell bodies. The staining is highly punctate suggesting possible receptor clustering. Therefore, it seems likely that the ChAT fibres are releasing acetylcholine which activates the ganglion cells via M_3 receptor mediated mechanisms.

Vimentin positive cells are also found in the lamina propria where they form a loose network (see Figure 6 A and 7 A). Some of these lamina propria interstitial cells are M_3-IR. It is also apparent in Figures 6 B and 7 A that vimentin positive cells can be found on the surface of the inner and outer muscle bundles. Here too M_3-IR can be seen. In addition, in 3 of 7 sham operated control bladders studied these M_3-IR cells were seen to be in close apposition lying between muscle bundles in structures which can be described as 'nodes' (Figure 6 D-F).

Bladders from animals with damage to the bladder neck

Bladders from animals with damage to the bladder neck were larger indicative of bladder wall hypertrophy. This is exemplified by examining the wall thickness of a normal bladder (Figure 7 A) with that of an operated bladder (Figure 7 B): 6 mm compared to 12 mm. There are also major differences in the number and distribution of vimentin positive cells. In obstructed bladders there is an increased density of vimentin fibres in the lamina propria and surrounding the muscle bundles, particularly the outer muscle bundles. In addition, there appears to be an increase in the M_3-IR in the lamina propria and outer muscle layer (Figure 7 B). These observations are elaborated in Figures 8 and 9.

In the bladders from obstructed animals the sub-urothelial interstitial cells persist and remain strongly M_3-IR. Little difference was noted in this cell layer in comparison to control bladders. However, there was a higher density of vimentin positive interstitial cells within the wall region between the sub-urothelial interstitial cells and the inner muscle layer. These cells are shown in

Figure 8 A and D. Figure 8 C shows a region of the section in Figure 8 B at higher magnification. These diffuse lamina propria interstitial cells are M_3-IR and appear to extend complex bifurcating processes. This network of lamina propria interstitial cells appears to be continuous with vimentin positive cells which run over the surface of the muscle bundles and within the muscle bundles (Figure 8 D). Examples of nodes are also shown in Figure 8 E and F.

As noted in Figure 7 B there is a high density of vimentin positive structures in the outer muscle layers of the OB-bladders. Further examples of this are shown in Figure 9. Cell bodies of these vimentin positive cells can be found in groups or 'nodes'. These nodes are found in close apposition of several muscle bundles. The cell bodies of these interstitial cell nodes are associated with M_3-IR. Vimentin positive cells are also apparent within the muscle bundles. Regions of the bladder wall can be found where trabeculae are seen with a high density of vimentin positive cells. Such trabeculae are often found in close apposition to others with few vimentin positive structures (see Figure 9 E-G). The intra-muscular vimentin positive cells can be seen to send processes to and be in contact with the vimentin positive structures lying on the surface of the trabeculae. In addition, these intra-muscular vimentin positive cells are M_3-IR (see Figure 9 F and G)

Discussion

In the present study we used an antibody which appears to detect M_3 type muscarinic receptors in the bladder wall. It has to be emphasised that the possibilities remain that the antibody exhibits some non-specific binding and also that it might also recognise other muscarinic receptor iso-forms. For these reasons the structures recognised by the antibody are described as having muscarinic receptor type 3 like immuno-reactivity (M_3-IR). This discussion is presented on the basis that the antibody identifies cells expressing muscarinic receptors. The reliability of this assumption may be increased since there are functional data to suggest the presence of muscarinic receptors on different cell types in the bladder wall.

It must also be pointed out that the present data are derived from guinea pig bladder. Morphologically the guinea pig bladder has many similarities with the human bladder (Dixon *et al.* 1983; Gabella 1990; Smet *et al.* 1996a; Smet *et al.* 1996b; Zhou and Ling 1998). In addition, pathologies such as that produced by partial outflow obstruction are similar to that seen in man (Mostwin *et al.* 1991). Thus, the model has value in unravelling the complications of the human situation.

In the normal bladder M_3-IR can be identified on different structures. M_3-IR is associated with the smooth muscle cells and this may represent the muscarinic receptors that are known to play a central role in the cholinergic parasympathetic post-ganglionic activation of the detrusor involved in the voiding contraction. The presence of M_3-IR on nerve fibres, ganglionic cell

bodies and interstitial cells suggests roles for muscarinic receptors in addition to the control of the voiding contraction.

There appears to be M_3-IR associated with a population of nerves fibres in the sub-urothelial space and in the muscle layer. Specific physiological roles for these potentially different nerve types have yet to be determined. At least two types of sub-urothelial sensory fibres have been identified in the guinea pig bladder: one type expressing ChAT and the other cgrp, substance P and the 200 kD neurofilament protein (Gillespie *et al.* 2006). It has been suggested that these fibres send collaterals to the sub-urothelial ganglionic network contributing to local reflexes in the bladder wall. It is possible that one or other of these nerve sub-types expresses M_3-IR. The presence of nerves expressing muscarinic receptors points to a possible regulation of their firing by cholinergic stimuli. Muscarinic receptors have been suggested to be present on pre-synaptic nerve endings in the bladder (Kim *et al.* 2005). M_1 receptors are thought to be facilitatory while M_2 and M_4 inhibitory (Somogyi and de Groat 1999). A role for M_3 receptors has not been demonstrated. Physiologically, the source of acetylcholine which might activate these nerves is not known. It may come from the cholinergic nerves themselves: a form of auto-excitation or inhibition. Alternatively, the acetylcholine may originate from the other structures such as the urothelium. It has been shown that, upon stretch, the urothelium of the human bladder releases acetylcholine (Yoshida *et al.* 2004). Thus, as with urothelial stretch induced ATP and NO release, acetylcholine released in this way may exert a modulatory role influencing afferent nerve activity.

M_3-IR and the presence of ChAT fibres associated with the intra-mural ganglia suggest that the ganglionic network has a presumably sensory cholinergic input. The precise role these ganglia play in the bladder is not known but it has been speculated that they are involved in local reflexes and that they are the site of integration of excitatory and inhibitory inputs (Lagou *et al.* 2005; Gillespie *et al.* 2006). The presence of different nerve types has also been noted in the muscle layers. Here, there are fewer insights into the possible physiological relevance of the different types of nerve but they may also be involved in the local reflexes alluded to above (Gillespie *et al.* 2006).

Perhaps the most unexpected observation in the normal bladder was the presence of M_3-IR associated with sub-urothelial interstitial cells and surface muscle interstitial cells. A brief note of this has appeared previously (Gillespie *et al.* 2003). The functional role of the sub-urothelial interstitial cells is not known. These cells are responsive to NO and demonstrate an NO dependent rise in cGMP. The basal layer of the urothelium expresses neuronal nitric oxide synthase and so can produce NO (Gillespie et al. 2005). Also, endogenous NO has been shown to activate the sub-urothelial interstitial cells (Gillespie *et al.* 2006c). Thus, the sub-urothelial interstitial cells are clearly able to respond to urothelial derived signals. The presence of muscarinic receptors suggests other possible mechanisms leading to the activation of these cells.

In the normal bladder there is a network of vimentin positive cells that surrounds the muscle bundles in the inner and outer muscle layers (Brading and McCloskey 2005; Drake *et al.* 2006). There may be different types of cells associated with the outer and inner muscle bundles. In the outer muscle layer these cells respond to NO with a rise in cGMP (Gillespie *et al.* 2004; Gillespie *et al.* 2006b) a situation also seen in the mouse (Lagou *et al.* 2006). The presence of M_3-IR on this network of cells suggests the possibility of a cholinergic regulation of their function. Experiments using the guinea pig isolated whole bladder preparation have demonstrated cholinergic mechanisms involved in the activation of phasic contractile activity. The properties of this phasic activity are consistent with the hypothesis of a cholinergic driven pacemaker mechanism affecting the frequency of the activity and a cholinergic dependent component involved in the distribution of the activity and the augmentation of its amplitude (Gillespie *et al.* 2004; Finney *et al.* 2007). The observations presented here provide circumstantial support for the concept that the interstitial cell network associated with the muscle layers are involved in the generation of phasic activity. The M_3-IR on the interstitial cells may represent either the pacemaker element or the regulation of the distributive element.

Phasic activity occurs in the normal bladder in vivo: non-micturition activity. Such activity has been suggested to play a central role in a motor/sensory system involved in the generation and regulation of bladder afferent nerve activity and sensation (Starling 1905; Gillespie 2005a). Evidence is thus accumulating in vitro to link cholinergic driven interstitial cells with phasic activity and in vivo to link phasic activity with the generation of sensation. Thus, interstitial cells may have key role to play in sensory mechanisms in the bladder.

In the present study, bladders from animals which had previous surgical damage to the bladder neck show distinct differences compared to control. The finding that there is loss of nerve fibres from the muscle have been reported previously (Charlton *et al.* 1999; Drake *et al.* 2000). This denervation must have profound effects on the properties and control of the bladder wall. Exactly what these changes in function are is not known. It is also apparent from this study that there are changes in the number and distribution of vimentin positive M_3-IR interstitial cells. There appears to be little change to the network of sub-urothelial interstitial cells but the amount of interstitial cells distributed in the lamina propria and within the muscle layers seems to increase. In addition there also seems to be an increase in the interstitial cells sham operated bladders. These cells have M_3-IR and may therefore be regulated by cholinergic mechanisms. A recent study has examined the number and distribution of cGMP identified interstitial cells in bladders from animals with previous damage to the bladder neck (de Jongh *et al.* 2007). In this work, nodes of cGMP positive cells were also described associated with the outer muscle bundles. It was also shown that, despite denervation of the muscle, nerve profiles were associated with these nodes. Thus, it is possible that the nodes are innervated

and that this might be a means of activating the cholinergic receptors on their surface resulting in regulation of the interstitial cell network.

Summaries of the major observations on vimentin positive and M_3-IR cells in both sham operated and obstructed bladders is shown in Figure 10 A and B. In the sham operated bladder there is a growing awareness that the physiological processes in the bladder wall rely on complex interactions between different systems and cell types. Each of these systems (urothelium and urothelial modulation of afferent nerves, the sub-urothelial system, the ganglionic network and the network of interstitial cells) has the possibility to interact. The present observations also demonstrated the possibility that within each system there may be a role for muscarinic receptors, specifically M_3. The dramatic changes in these systems induced by bladder neck damage and the alterations to the functional properties of the bladder suggest links between specific structures and altered function. It has recently been shown that the isolated bladders from animals with previous damage to the bladder neck demonstrate large phasic contractions and that the bladders appear to have an increased sensitivity to applied cholinergic stimuli (de Jongh *et al.* 2007b). The loss of intra-mural ganglia and the altered number and distribution of M_3-IR muscle interstitial cells may be linked to these functional changes. It has been speculated that one of the pathologies of the bladder could arise following damage to the bladder neck, the loss of intramural nerves and the up-regulation of the system of interstitial cells. Since the interstitial cell network may be involved in the generation of phasic activity and the modulation of sensation its potential role in the sensation of urgency and frequency are obvious. The present observations add to this concept by suggesting the possibility that the interstitial cell network may be regulated by cholinergic mechanisms. This increased activity could contribute to increased motor/sensory activity. The cholinergic component of this modulated sensory system being up-regulated by the pathology and, possibly, one of the therapeutic targets for the anticholinergic drugs.

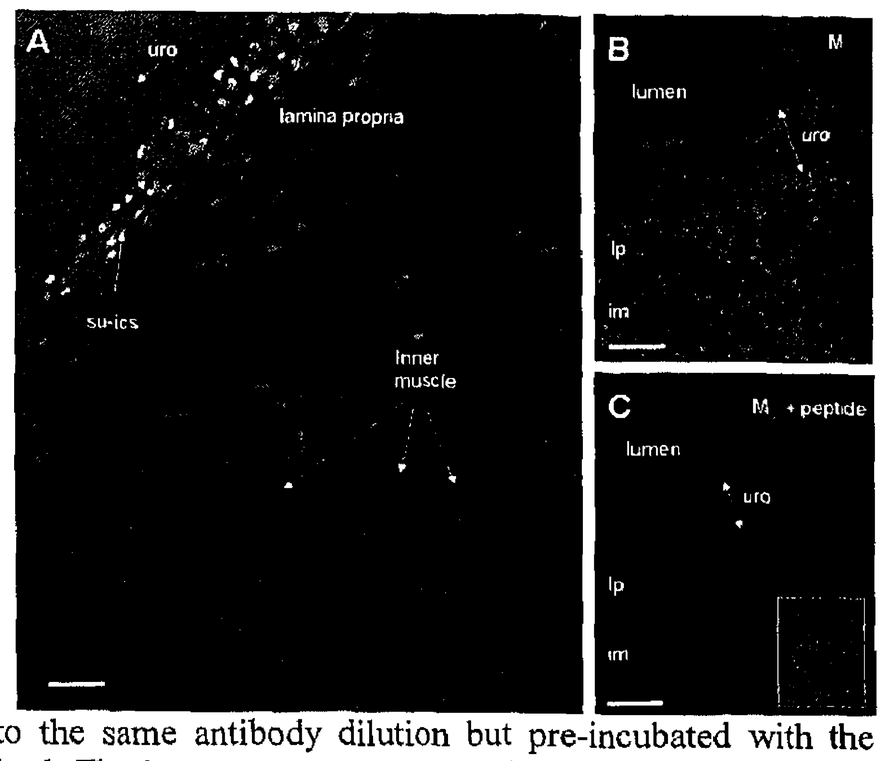

Figure 1. Localisation of M_3 antibody binding within the lamina propria of the guinea pig bladder. A shows a section of the luminal surface of the bladder wall. The urothelium (uro), lamina propria (lp) and the inner muscle (im) layer are identified. Cells showing M_3 immuno-reactivity (M_3-IR), are seen in the layer immediately below the urothelium in the layer of cells described as sub-urothelial interstitial cells (SU-ICs). Staining, although weaker, is seen associated with the muscle bundles of the inner layer. B and C are adjacent sections from the same bladder. B was exposed to the M_3 antibody while C was exposed to the same antibody dilution but pre-incubated with the peptide to which the antibody was raised. The images were captured using the same gain on the low light level digital camera. The inset in C shows an enhanced image of C to show the content of the section. The absence of staining in C strongly suggests that the M_3-IR is likely to be the M_3 receptor. Calibration bars: 80 μm in A and 110 μm in B and C.

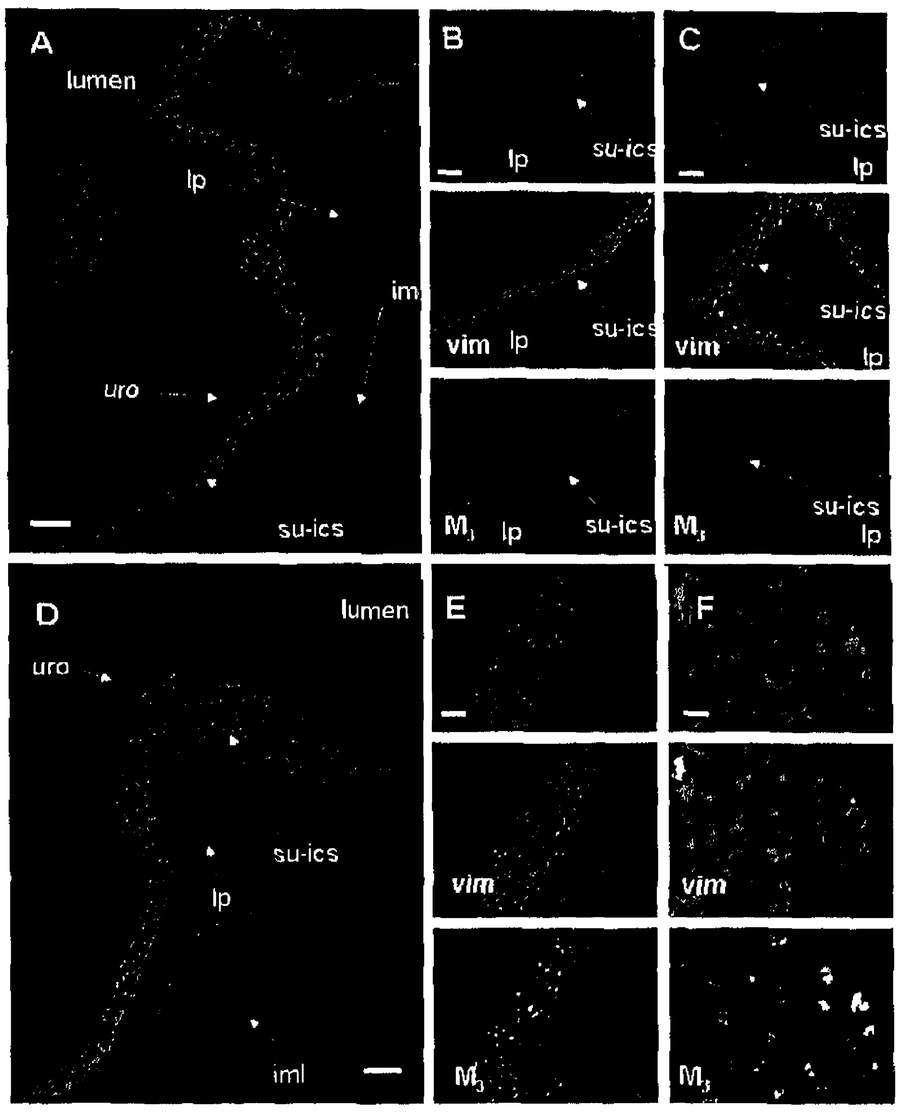

Figure 2. Co-localisation of M_3-immunoreactivity (IR) with vimentin in the sub-urothelium and lamina propria. The colour panels show sections double labelled with antibodies to M_3 (red) and vimentin (green). In A the lumen, urothelium, sub-urothelial layer and the inner muscle layer are identified. B and C show regions of the image in A but with the individual images of the vimentin staining and the M_3-IR. The layer of sub-urothelial interstitial cells is strongly positive for vimentin. M_3-IR is also seen within this layer located within the vimentin positive cells. Comparison of B and C shows that there are variations in the density of the M_3-IR staining in nearby regions of the bladder wall: there appears to be less M_3-IR in B compared to C. Note that here is little M_3-IR within the lamina propria. D shows a section of the urothelium from a different bladder. E and F show regions of D at high magnification with the individual M_3 and vimentin images. E point out that all of the sub-urothelial interstitial cells are M_3-IR. F shows, at higher magnification, that most of the M_3-IR appears to be located in the central cell body of the SU-ICs. Calibration bars: 120 μm in A and D, 75 μm in B, C, and E and 15 μm and F.

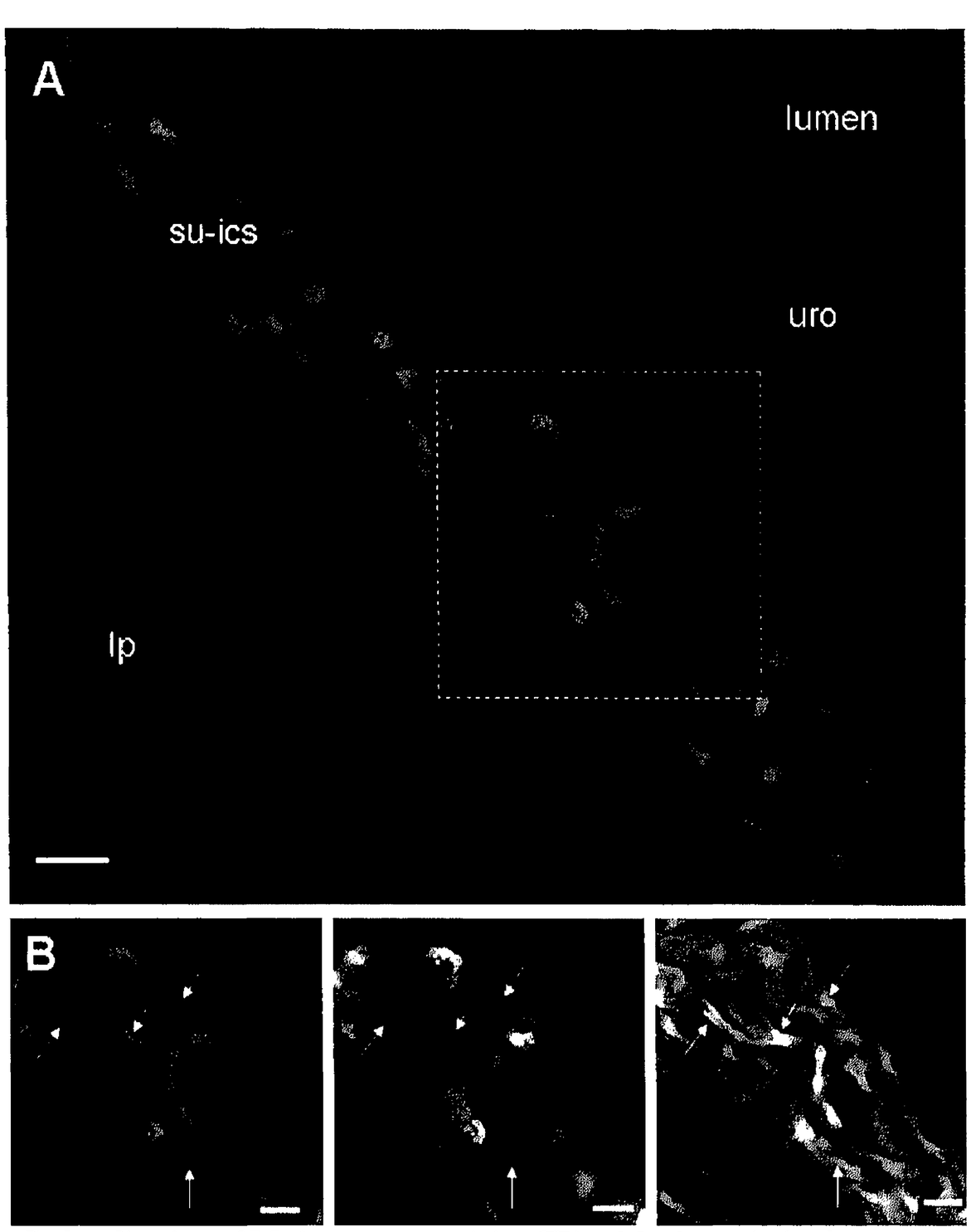

Figure 3. Co-localisation of M_3 immunoreactivity (IR) with vimentin in the processes of sub-urothelial interstitial cells. A shows a section double labelled with antibodies to M_3 (red) and vimentin (green). The lumen, urothelium, sub-urothelial layer and lamina propria are identified. Note the presence of cells in the lamina propria which have M_3-IR. The region highlighted by the square is shown in B along with the separate images of M_3-IR and vimentin. The arrows identify weekly staining M_3-IR associated with the processes of the sub-urothelial interstitial cells. Calibration bars: 50 μm in A and 25 μm in B.

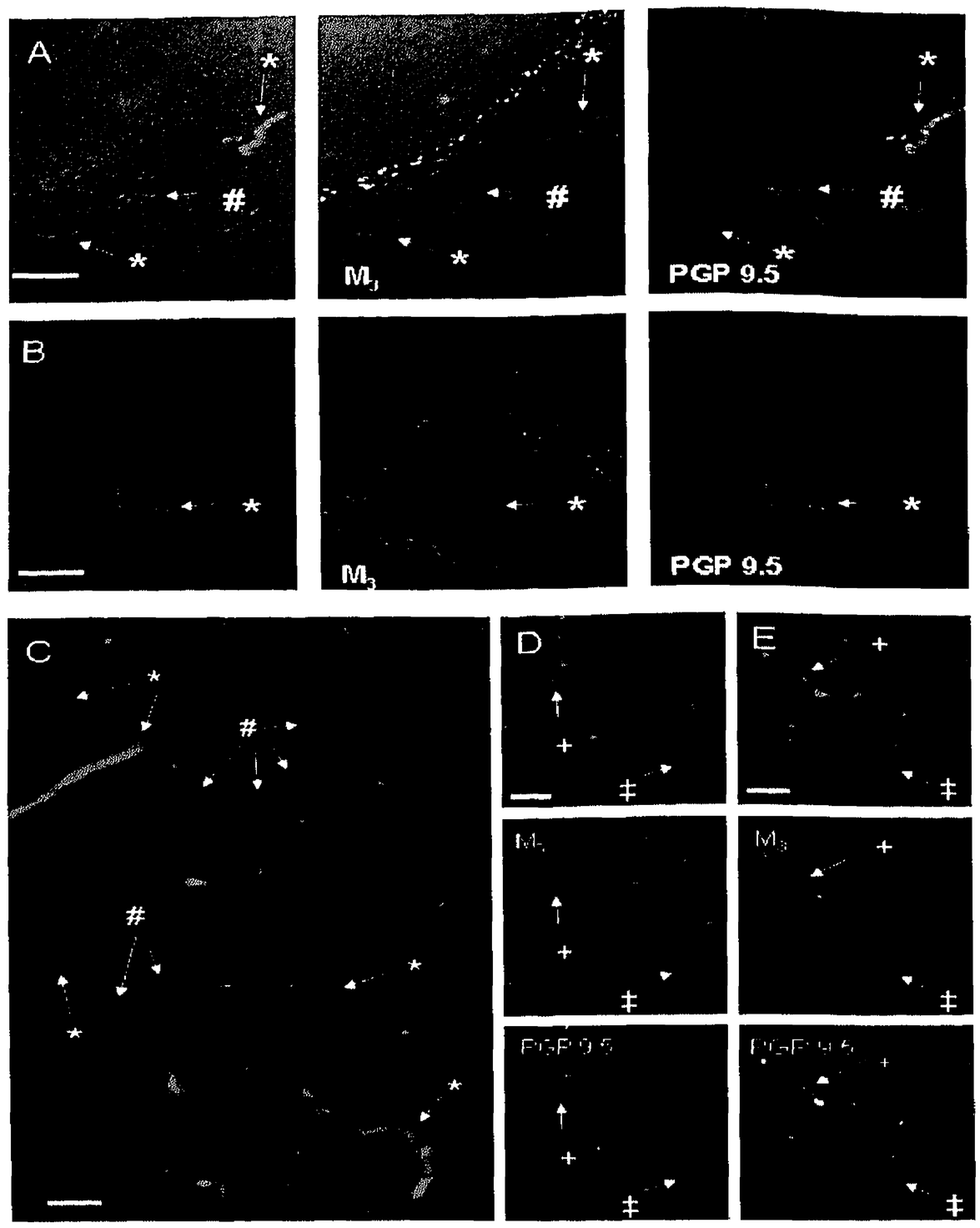

Figure 4. Illustration of M_3 immunoreactivity (IR) and nerves in the lamina propria and muscle. The colour panels show sections stained with antibodies to M_3 (red) and the non-specific nerve marker PGP9.5 (green). A and B show the colour images with the composite images for M_3-IR and PGP9.5 shown on the right. A shows nerve fibres in the lamina propria as determined by PGP9.5 staining (far right hand panel). The fibres marked (*) are also M_3-IR. However the fibre marked (#) is not M_3-IR. B shows images from a different bladder illustrating a nerve fibre which is M_3-IR. In C the section is taken from the outer muscle layer. Nerve fibres are easily recognised (*) and M_3-IR is associated with the smooth muscle cells (#). D and E illustrate regions of the image in D with the accompanying M_3-IR and PGP9.5 images. In the colour panels nerve fibres are easily recognised. Examination of the images showing M_3-IR reveals that there are nerve fibres which are M_3-IR (+) while there are fibres which are not (‡). Thus there appear to be two different types of nerve fibre M_3-IR positive (M_3-IR^+) and M_3-IR negative (M_3-IR^-). Calibration bars: 120μm in A and B, 40μm in C and 50μm in D and E.

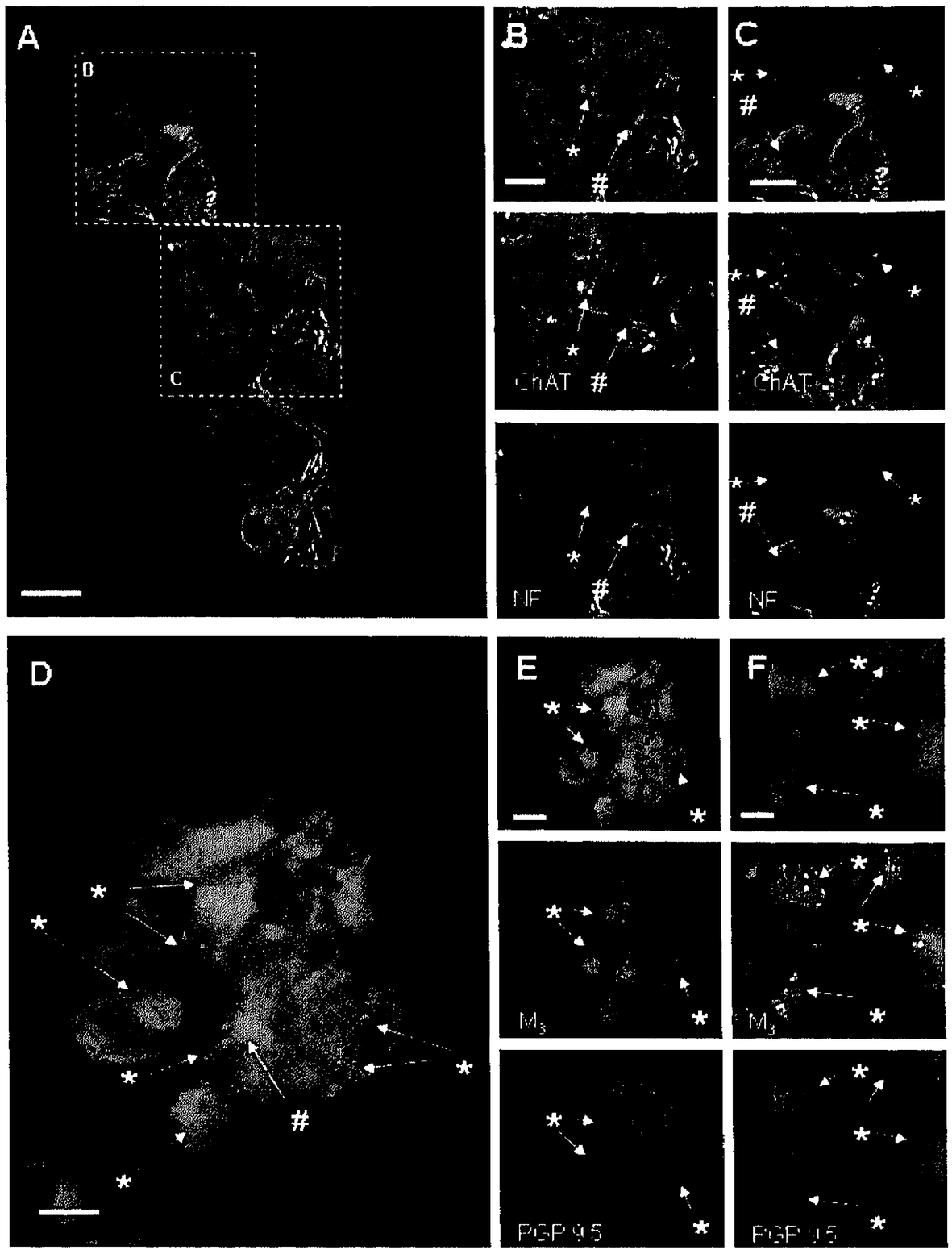

Figure 5. Intra-mural ganglion with possible cholinergic and peptidergic inputs. A shows a section containing an intra-mural ganglion. The section is stained with an antibody to the enzyme choline acetyl-transferase (ChAT, red) and neuro-filament protein (NF) (green). Nerve cell bodies can be seen and ChAT positive terminals are apparent on the cell bodies. B and C show regions of the image in A with the accompanying ChAT and NF images. In B the ChAT positive terminals are readily seen (*) in addition to ChAT positive fibres with varicosities (#). D shows an intramural ganglion stained with antibodies to the non-specific neuronal marker PGP 9.5 (green) and the M_3 receptor (red). E illustrates the same ganglion but with the individual images for PGP 9.5 and M_3-IR. The punctuate localisation of M_3-IR is clearly seen. F illustrates a ganglion from a different bladder where the punctuate distribution of M_3-IR is also apparent. Calibration bars: 40μm in A, 30μm in B and C and 40μm in D, E and F.

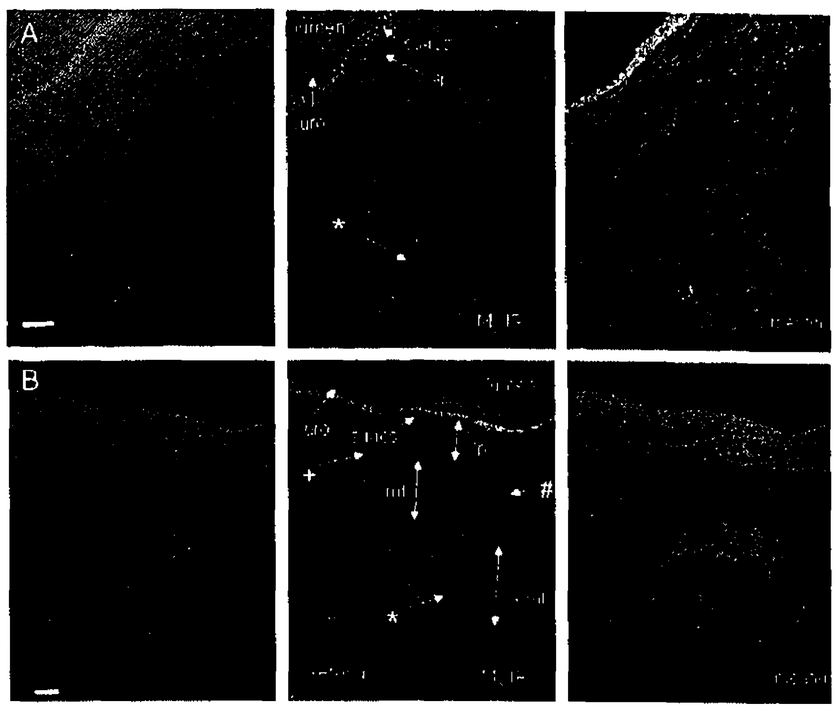

Figure 7. Full thickness sections of a sham operated bladder and a bladder with previous surgical intervention to restrict the bladder neck (note the difference in scaling). In each of the colour panels vimentin is shown green and M_3-IR red. A shows the sham operated bladder and B the bladder with previous damage to the bladder neck. The individual images of M_3-IR and vimentin are also shown. The lumen, serosa, urothelium (uro), sub-urothelial interstitial cells (SU-ICs) and lamina propria (lp) are indicated by arrows. In the bladder neck operated animals the inner muscle layer (iml). and outer muscle layer oml are also shown. Discrete M_3-IR is seen in the lamina propria and the outer muscle layers. Calibration bar: 100μm in A and 110μm in B

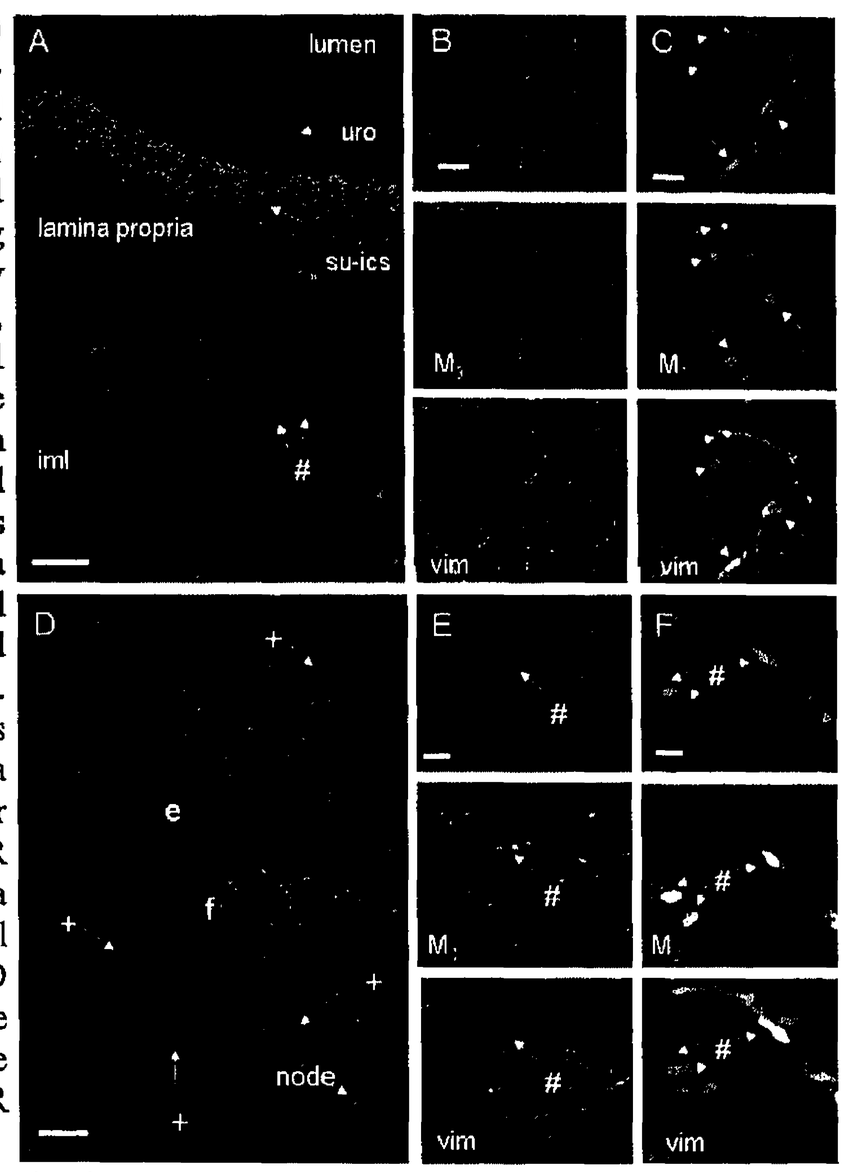

Figure 8. M_3-immunoreactivity (IR) and vimentin positive cells in the sub-urothelial layer, lamina propria and muscle layers in bladders from obstructed animals. The coloured sections illustrate the double labelling with vimentin (green) and the antibody to the M_3 receptor (red). In A the lumen, urothelium (uro), the sub-urothelial cell layer, lamina propria and inner muscle layer (iml) are marked. M_3-IR is seen in structures within the sub-urothelial cell layer and in the lamina propria. B shows a region of the lamina propria from a different bladder. The upper panel shows the combined stained image and the lower panels the individual images. A loose layer of vimentin positive cells is seen in the lamina propria. C shows a region of the images in B but at higher magnification. Vimentin positive/M_3-IR cells are seen. These cells have a distinctive morphology: a round cell body with long ramifying processes. D shows a network of vimentin positive cells lying over the surface of muscle bundles in the mid muscle layer. M_3-IR is seen associated with the cell bodies of these structures. Vimentin and M_3-IR is also seen in structures within the muscle bundles. E and F show regions of the image in A at higher magnification along with the accompanying images of vimentin and M_3-IR. The localisation of the M_3-IR to the cell bodies of the vimentin positive structures is clear. + and # show M_3-IR and vim^+ structures respectively. Calibration bars: 90μm in A and D, 50 μm in B and E, 20 μm in C and 15 μm in F.

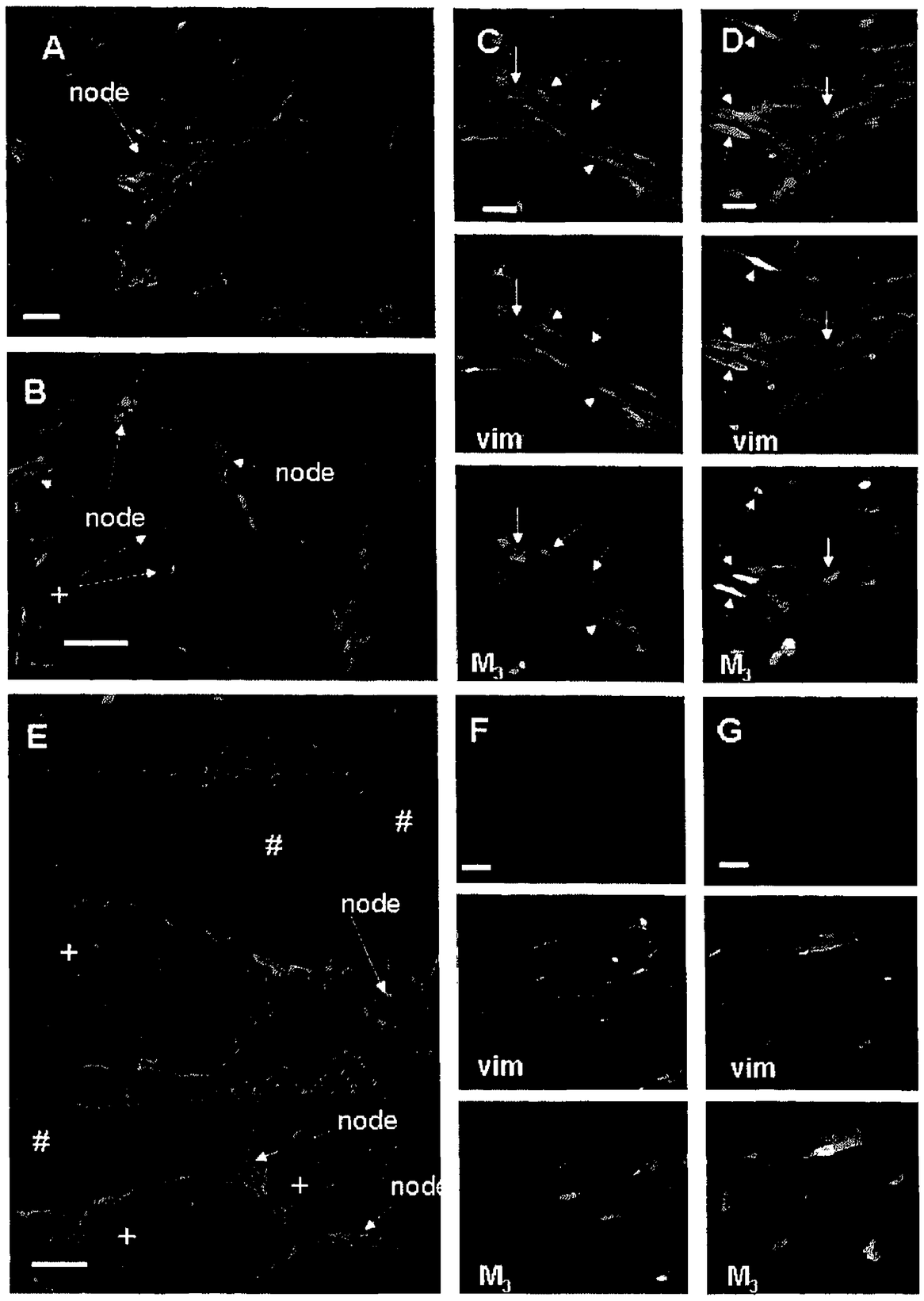

Figure 9. 'Nodes' of vimentin positive and M_3-immonoreactive (IR) cells in the outer muscle layers of an obstructed guinea pig bladder. Panels A, B and E show examples of sections doubled stained with vimentin (green) and the M_3 antibody (red). Bundles of vimentin positive fibres are seen to run between the muscle bundles.The cell bodies of these structures appear to be collected together to form nodes. Structures associated with the cell bodies of these nodes are M_3-IR. Note the presence of vimentin positive and M_3-IR in structures within the muscle bundles. C, D, F and G show regions of these images at higher magnification along with the associated individual images of the vimentin and M_3-IR. The localisation of the M_3-IR to the region of the nucleus and cell body of these cells is readily seen. Panel E illustrates the presence of two categories of muscle bundle: those with numerous intra-muscular interstitial cells (+) and those with few intestinal cells (#). F and G show examples of the intra-muscular interstitial cells which are M_3-IR. Calibration bars: 80μm in A, B and E and 15 μm in C, D and F.

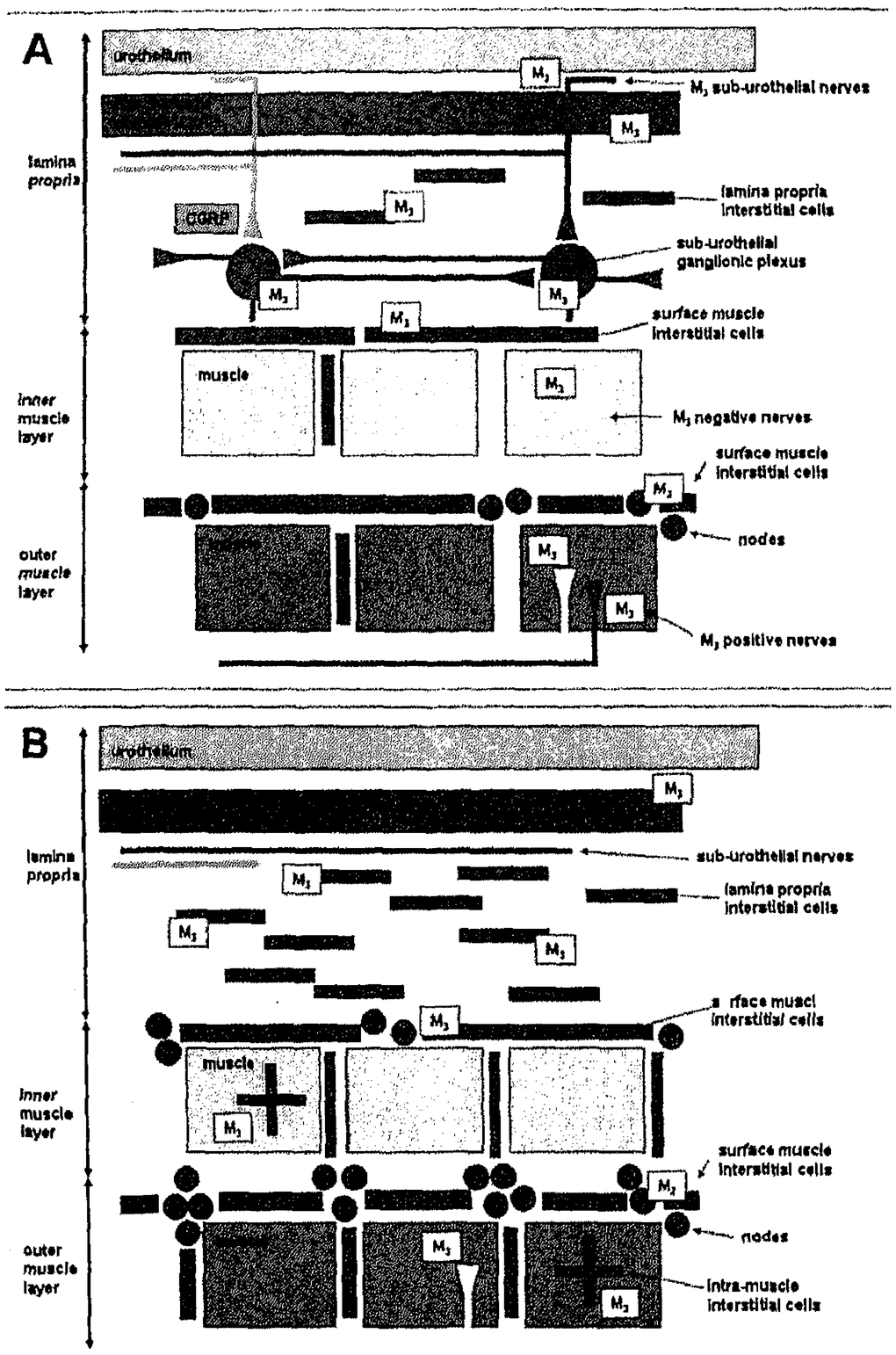

Figure 10. Schematic diagrams illustrating the observations made on the localisation of M_3-IR to cells and structures in the wall of normal bladders and bladders with previous surgical intervention to restrict the bladder neck. A shows the normal bladder. M_3-IR is located within sub-urothelial interstitial cells, cells in the lamina propria, nerves in the lamina propria, on the intra-mural ganglia, on superficial muscle interstitial cells in the inner muscle layer, on interstitial cells of the outer muscle layer, on small nodes and on a population of nerves in the muscle bundles. B shows the situation in the obstructed bladder (OB). M_3-IR is located to the same cell types as in the control bladders but there are be differences in the number and distribution of these cells. In the OB bladders there seem to be more M_3-IR cells dispersed within the lamina propria. M_3-IR positive superficial muscle interstitial cells appear to be more abundant particularly in the outer region. There are also prominent nodes of M_3-IR positive cells. Muscle bundles are found which have a high incidence of intra-muscular M_3-IR positive cells while others have few such cells.

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Chapter 8

Summarizing Discussion and Conclusions

Disregulation of the control mechanism of the urinary bladder presents a considerable clinical problem. Therefore, it receives a great deal of attention from both clinicians and basic scientists. Because experiments in humans raise legal, moral and ethical dilemmas, such studies are inherently limited. Therefore in the last century, experimental work on human bladder function has focused primarily on *in vivo* cystometric investigations and structural studies of biopsy material. However, it is impossible to acquire a significant amount of bladder tissue from healthy normal subjects for investigating the cellular structure and the locations of different receptors in the bladder. Thus, basic science has resorted to the use of animal models to gain insights into the principles governing integrated control of the lower urinary tract and its cellular components.

The guinea pig bladder shows significant structural similarities with the human bladder and obstructed guinea pig bladders show similar cystometric changes as patients suffering from the overactive bladder syndrome [1]. Hence, this is a good model to study changes in bladder structure after outflow obstruction. Bladder outflow obstruction results in many structural and functional changes in the bladder wall: the smooth muscle is more active and more sensitive to acetylcholine, the urothelium thickens, there is hypertrophy of the smooth muscles and a punctuate loss of the motor nerves [2, 3]. It has recently been suggested that these changes are triggered not by the obstruction *per se* but by the local damage that occurs to the bladder neck following surgery, which can be exacerbated by physical obstruction [4]. Such local damage results in global changes in the bladder wall.

We investigated the regional differences in sensory innervation and suburothelial interstitial cells (ICs) in the bladder neck and urethra (**chapter 3**). For a long time it has been known that obstruction to the bladder neck was associated with an increase in voiding frequency, an increase in bladder excitability and an increase in bladderwall thickness [5]. Experimentally, similar changes result from ligatures applied to the urethra that are put in place to reduce urine flow, an approach designed to mimic bladder outflow obstruction caused by conditions such as benign prostatic hyperplasia [3]. However, there are reports demonstrating that similar changes can occur in animals and humans where the bladder neck is only damaged and not obstructed [4]. In order to explore what structures could be damaged in the bladder neck we must have a detailed overview of the structure of the lower urinary tract in the region of the bladder neck. In this region, sensory nerves penetrate the urothelium and there is a dense area with ICs. The observation of an area densely populated with ICs in the bladder urethral junction (BUJ) adds to the complexity of ICs found in the lower urinary tract (LUT). ICs have been linked to sensory functions while other ICs are excitable and generate electrical activity and communicate via gap junctions with the smooth muscle to influence contraction [6]. Also some ICs are thought to generate pacemaker activity and influence phasic activity in the bladder [7]. There is a possibility that the

specialised cluster of ICs in the region of the BUJ might have sensory or pacemaker functions. In this region a reduction in the amount of muscle in the bladder wall is observed. It could be that this region is less rigid then adjacent regions with muscle and thus more compliant to distension. The ICs together with the sensory innervation in this region may be specialised to respond to distension with the generation of localised afferent nerve discharge. This could contribute to a specific and localised sensation.

Prostaglandins (PGs) are produced in both the urothelial and muscle layers of the urinary bladder in response to bladder distension [8]. PGs alter bladder activity by influencing voiding and smooth muscle contractility [9]. Regarding the changes in voiding frequency it has been hypothesized that PGs might act directly on the afferent nerves to modulate firing and so trigger micturition at lower bladder volumes [10]. With regard to a direct action on the muscle, it was pointed out that they could be co-released with acetylcholine at efferent nerve endings and so directly contribute to muscle excitation [11]. Alternatively, they might act indirectly on pre-synaptic motor terminals to affect the release of excitatory transmitters [12]. It has also been considered that PGs might inhibit acetylcholine-esterase or enhance myogenic bladder activity [13].
Using the isolated bladder model it was reported that exogenous PGs can modulate the autonomous activity [7]. The action of PGs could play a role in the motor component of a motor/sensory system involved in the generation of afferent firing and bladder sensation [14]. Thus, increased phasic motor activity would result in an increase in afferent discharges and, subsequently influence the point at which voiding is triggered. To further understand the function of PGs in the modulation of autonomous activity, it is essential to know where PGs are synthesized and where the receptors for PGs are located. PGs are synthesised by the two distinct enzymes: cyclo-oxygenase type I (COX I) and cyclo-oxygenase type II (COX II) [7]. COX I is constitutively expressed in the urinary bladder [7]. COX II expression has also been observed in the normal bladder, however the expression of this enzyme is strongly increased during inflammation of the bladder [15]. In this study we demonstrate that COX I-IR predominates within two general cell systems in the bladder wall: (i) cells within the basal and intermediate layers of the urothelium and (ii) within a population of ICs. These ICs are present throughout the sub-urothelial space of the lamina propria and extend over the surface of the muscle bundles, which make up the inner layers. It is interesting and important to note that no COX I-IR was seen within the muscle bundles indicating that, ICs in the guinea pig and not the smooth muscle cells are producing PGs. These observations raise intriguing questions regarding the mechanisms of action of PGs in the guinea pig bladder. One hypothesis might be that COX I in the basal urothelial cell layers is activated by bladder distension. The PGs produced here could diffuse over a relatively short distance to the sub-urothelial space in which sensory afferent nerve fibres are found.With this arrangement it would be explained

how PGs might modulate afferent nerves. However, the abundance of COX I-IR in the urothelium of the lateral wall and the relative paucity of afferent nerves indicates that this can not be the only role for PGs produced by the urothelium in the lateral wall.

The cells in the basal urothelium express both COX I and neuronal nitric oxide synthase (nNOS). Like PGs, nitric oxide (NO) is produced by the urothelium in response to stretch [16]. It is known in other cell systems, that PG production is influenced by NO and, conversely, that NO production is influenced by PGs [17]. Other signals also originate in the urothelium in response to stretch [18]. Specifically adenosine triphosphate (ATP) has been shown to be released and one of its actions is to influence afferent nerve firing [18]. There are also reports that acetylcholine is released from the urothelium [19]. Adding to this complexity, the ICs immediately below the urothelium, the sub-urothelial interstitial cells (SU-ICs) can be a possible location for further integration of urothelial derived signals. In the guinea pig and human these SU-ICs respond to both exogenous and endogenous NO with an increased synthesis of cyclic guanosine monophosphate (cGMP). There is evidence that these cells also express type 3 muscarinic receptor (M_3), purinergic receptor (P2Y6) and the type 2 prostaglandin receptor (EP2). Thus, the integrated output of PG, NO, ATP and cholinergic stimuli from the urothelium can further be integrated and modulated on the SU-ICs. The SU-ICs are in close relation with the network of ICs of the lamina propria continuing on the surface of the inner muscles bundles.

The complex scheme makes it possible to outline a mechanism to explain volume related events. Mechanical deformation of the urothelium in the lateral wall triggers complex cascade of interacting signals within the urothelial epithelium resulting in the release of signals into the sub-urothelial space. There, these urothelial signals are further integrated on the SU-ICs. Activity in the SU-ICs is then distributed to the muscle via the IC network resulting in the excitatory and inhibitory effects on the phasic activity.

Prostaglandin synthesis has been associated with the smooth muscle [20]. Since the surface muscle ICs (SM-ICs) show COX I-IR, these cells are responsible for synthesising PG in the muscle layer. The SM-ICs form a network on the surface of the muscle bundles. The possible role of this network is to generate and distribute signals leading to phasic contractions in the bladder wall. PGs may be involved in the generation and distribution of these signals.

Surprisingly, we found occsional UBH-IR in all types of interstitial cells of the normal bladder. The binding of ubiquitin to a protein targets a protein intracellularly for protein degradation [21]. UBH separates ubiquitin from its protein substrate affecting the fate of ubiquitinylated proteins, which results in the prevention of protein degradation [22]. UBH is therefore involved in the regulation of protein trafficking, and thus an indicator of cells undergoing major changes in structure, differentiation and phenotype.

We found occasional UBH-IR in all types of interstitial cells the normal bladder. The interstitium of the bladder urethral junction contained a dense population UBH-IR interstitial cells. This subtype of ICs must serve a specific function, however the nature of this function is not known. It may relate to the regulation of extra-cellular matrix or have a more dynamic functional role. In the lateral wall of both sham operated and obstructed guinea pig bladders we found an up-regulation of UBH in urothelial cells and a subset of ICs. These cells are one of the first cell types to show activation following damage to the bladder neck. These UBH positive ICs are located in the lamina propria (lamina propria interstitial cells; LP-ICs), on the surface of the muscle bundles (surface muscle interstitial cells; SM-ICs) and in the muscle coat (muscle coat interstitial cells; MC-ICs).

The LP-ICs and SM-ICs constitute a network of ICs extending throughout the bladder. These cells appear to be particularly activated following bladder neck damage. It is possible that an initial local activation could be rapidly propagated throughout the entire network resulting in activation of the entire network. Activation might then trigger changes to the other cellular system in the bladder wall resulting in the observed global pathology. It has been demonstrated that there is a sub-population of LP-ICs and SM-ICs that express the enzyme COX I and thus are able to produce prostaglandin [23]. The local release of prostaglandin may be involved in this system of propagation and activation, since the interstitial cells that show COX I-IR are upregulated following bladder outlet obstruction [15].

In conclusion, distribution of UBH in the obstructed bladder identifies a novel sub-set of ICs. These cells appear to form a network of activated and proliferating cells which extend through out the bladder wall.

When we started this thesis, the localisation of the M_3 receptor in the guinea pig bladder was not known. It has been demonstrated that M_3 knockout mice have a drastically changed bladder activation, but still appear to void normally [24]. It has been known for a long time that activation of the M_3 receptor results in activation of the detrusor [25]. For this reason it was assumed that the M_3 receptors were located on the detrusor muscle and that this was the site of action of the M_3 specific antagonists used in clinical practice [26]. Given the importance of M_3 receptors in the activation of bladder contractions, the concept arose that the cause of the OAB syndrome had to be in the bladder muscle. This resulted in the concept that the M_3 receptors play a role in the autonomous contractions [27]. Clinically, M_3 specific antagonists are effective in reducing symptoms of urgency and frequency [28]. However, it has become apparent that, at therapeutic doses, these drugs do not affect either the non-voiding contractions nor the voiding contractions [29]. Thus, possibly the site of action of the M_3 specific anticholinergic drugs is not on the detrusor. For understanding the working mechanism of M_3 antagonists it is essential to identify the cell systems on which the M_3 receptors are located. It has been hypothesised that substances released from the urothelium act on the LP-ICs,

whom in turn respond by a contraction and so distorting and activating the adjacent afferent nerves [30]. However we found that not the LP-ICs show M_3-IR, but the SU-ICs (**Chapter 6**). This sub-urothelial layer is poorly innervated with sensory fibres, thus our observations suggest that acetylcholine (ACh) derived from the urothelium may have an effect on the SU-ICs, in addition to a possible activation of sensory/afferent fibres in the sub-urothelium.

Antibodies are used in order to identify specific structural features. The use of antibodies has several disadvantages. For example the structure of specific peptides which an antibody is raised against may be slightly different in different spiecies. A small structural difference in a peptide in the area where the antibody was raised against may result in the lack of specific staining. Thus when an antiby shows specific immunoreactivity in one spiecies it may not work in different spiecies. Next to this difficulty there is the fact that there are differences in the stucture of the bladder wall between spiecies. Some celltypes which show specific immunoreactivity in one spiecies, might not be present in another spiecies. For these reasons one should be careful in extrapolating immunohistochemical results from one spiecies to another.
As already recapitulated before, bladders from animals, which had previous surgical damage to the bladder neck, show distinct differences, compared to control. The denervation and the loss of a ganglionic network in the guinea pig must have profound effects on the properties and control of the bladder wall. We observed that there are changes in the number and distribution of M_3 receptors on the ICs in denervated bladders. There appears to be little change to the network of SU-ICs but the number of ICs distributed in the lamina propria and within the muscle layers is increased. In addition, there also seems to be an increase in the ICs nodes in bladders with previous surgicical damage to the bladder neck (**Chapter 7**). These ICs express M_3 receptors and therefore may be regulated by cholinergic mechanisms. Despite the denervation of the muscle bundles, nerve profiles are associated with these nodes, indicating that they are innervated. This might indicate an activation of the cholinergic receptors on their surface resulting in modulation of the IC network. These bladders demonstrate large phasic contractions and appeared to have an increased sensitivity to cholinergic stimuli [4]. The loss of intra-mural ganglia and the upregulation of M_3 receptors in muscle ICs may be linked to these functional changes. Since the IC network may be involved in the generation of phasic activity and the modulation of sensation its potential role in the increased sensation of urgency and frequency are obvious. The observation that M_3 receptors are located on SM-ICs adds to this concept by suggesting that the network of ICs may be regulated by cholinergic mechanisms. This increased activity could contribute to increased motor/sensory activity. The cholinergic component of this modulated sensory system is up regulated by the pathology and, may be one of the therapeutic targets for the anticholinergic drugs.

The studies in this thesis suggest that there is a complex interaction between the urothelium and ICs in the sub-urothelium. The urothelium has been shown to release PG [8], ATP [18], NO [18] and ACh [31] primarily in response to stretch. This release is thought to be the initial step involved in a system for detecting bladder volume. As the bladder fills the urothelium is stretched and substances are released which results in the activation and modulation of afferent nerves [32]. There is experimental evidence demonstrating ATP modulation of afferent nerve firing and indirect evidence for the involvement of NO and ACh in the modulation of the afferent limb of the micturition reflex [32]. PG, ATP, ACh and NO appear to be released from the urothelium of the lateral wall and dome. In these regions the density of afferent nerves is low. Therefore, is seems likely that substances released in these regions sub-serve functions other than direct neuro-activation or neuro-modulation.

For example, it has been suggested that there are specialized cells in the lamina propria, which lie in close proximity to afferent nerve fibres. These LP-ICs respond to ATP, which has led to the idea that the LP-ICs contract in response to ATP so distorting and activating the adjacent afferent nerves. Although an interesting concept, there is no direct experimental evidence for the operation of such an arrangement in the bladder wall. Based on the original description of LP-ICs it seems unlikely that the SU-ICs are the same sub-type as the LP-ICs. A further aspect, suggesting that LP-ICs and SU-ICs are different, is that the SU-ICs layer in the lateral wall is poorly innervated with sensory fibres.

The absence of nerves in the sub-urothelial layer further suggests that the SU-ICs do not have an efferent supply and so are not under the influence of any significant neural control. The SU-ICs most likely will be activated by signals from the urothelium. The network of SU-ICs extends from the bladder base, over the lateral wall and into the dome. The cells, which lie in the sub-urothelium of the bladder, were first described by Smet as a population of cells that responded to exogenous NO with a rise in cGMP [33]. The physiological role of these cells and the significance of this responsiveness were not investigated. Subsequent work has confirmed the sensitivity of this cell layer to NO and shown that they lie in close proximity to cells in the basal urothelium which express nNOS [3]. This suggested that there is a transfer of signals between the urothelium and SU-ICs.

It has been shown that, in the guinea pig, the basal and intermediate cells of the urothelium express COX I [23]. Therefore these cells are likely to be responsible for the production of PGs in the urothelium. The cellular targets for the PGs are not known but there is preliminary data suggesting that the SU-ICs express the type 2 PG receptor (EP2) [34]. In other tissues the EP2 receptor is linked to adenylate-cyclase and, when activated, generates a rise in intracellular cyclic adenosine monophosphate (cAMP). Thus, another diffusible urothelial derived signal, PGs, has the potential to interact with the SU-ICs and to generate a second cascade of intracellular signals.

This thesis shows that these SU-ICs also express M_3 receptors suggesting that they can respond to ACh. It has been shown that ACh is released from the urothelium in response to stretch [35]. Data from human studies suggests that the outer cells of the urothelium express the enzyme choline acetyltransferase (ChAT) and are thus responsible for the synthesis of ACh [35]. For this reason, the M_3 receptor on the SU-ICs is most likely activated by ACh secreted from the urothelium in response to stretch. The M_3 receptors belong to the sub-set of muscarinic receptors that couple to G proteins and when activated result in a rise in diacylglycerol and inositol-trisphosphate (IP3) [36]. The IP3 generated might subsequently initiate the release of intracellular calcium, which results in further activation of signalling pathway(s) in these cells.

Recent evidence strongly suggests that the ICs of the detrusor are heterogeneous and form a network [3]. These cells might subserve the functions of pacemaker and of a coordinating network controlling contraction waves in the detrusor. In the guinea pig and mouse, the responsiveness of ICs to NO with the subsequent increase in synthesis of cGMP has been used to identify subtypes and their distribution. In the guinea-pig three types of ICs have been identified associated with the outer muscle layer: cells running on the outer margin of the bladder wall (MC-ICs), cells on the surface of the muscle bundles (SM-ICs) and cells within the muscle bundles (intramuscular interstitial cells; IM-ICs) [37]. In the mouse there appear to be only two types of IC associated with the outer muscle layers: IM-ICs and SM-ICs [38]. In both guinea pig and mouse the IM-ICs and SM-ICs come into close contact with nerve fibres, suggesting that activity in these cells might be under neural control. The concept that is evolving is that cholinergic nerve-mediated activity in the network of ICs is transferred to the underlying muscle bundles resulting in coordinated episodic waves of contractile activity. There is a report showing that NO selectively reduced the phasic activity in the isolated mouse bladder [39]. In the mouse bladder ICs are the major cell type sensitive to NO , which suggests that these cells play an integral part in the generation of phasic activity.

The autonomous bladder hypothesis suggests that the muscle ICs are involved in the activation and co-ordination of complex phasic activity (autonomous activity). This activity is the motor component of the motor sensory system [40]. The amplitude and frequency of the autonomous activity is increased by cholinergic agonists and ATP. If this type of activity is generated within an IC network this points out that there are M_3/ATP activated pacemaker cells linked to a distributed network. Possibly the SU-ICs play a role in the pacemaker function. The location and nature of a potential pacemaker mechanism is not known. In the guinea pig, a recent study showed the presence of a suburothelial ganglionic network [41]. The neurones in these ganglia are contacted by different types of nerve fibre. There is evidence for a suburothelial ganglionic network with the potential to receive inputs from the urothelium and adjacent ganglia and which has outputs to the muscle layers [41]. It has been speculated

that such a network might play a role in the local reflexes in the bladder wall linking urothelial distortion to phasic contractile activity. In the context of this discussion it is notable that the suburothelial ganglion cells receive inputs from two types of nerve fibres that are likely to be cholinergic suggesting that the ganglionic cells express cholinergic receptors [41]. Application of exogenous muscarinic agonists might be expected to activate these ganglia. If these ganglia are associated with reflex activation of phasic activity then the direct activation with an exogenous muscarinic agonist will also generate phasic activity. Therefore, there is evidence to suggest a role for the suburothelial ganglionic network, the pacemaker, and the different types of ICs, a heterogeneous network carrying pacemaker activity to the muscle layer.

In relation to this it is worth speculating about the origin of sensation in the bladder. It has been pointed out that sensations of bladder volume increase appear to be different at different stages of filling [42]. In the early stages of bladder filling weak sensations are felt in the general region of the bladder and lower abdomen [43, 44]. However, as the volume increases these sensations become more intense, sometimes described as urgency, and appear to come from lower down in the LUT in the region of the bladder neck and urethra [44]. These sensations are then mostly felt in the region of the external genitalia. A growing number of observations suggest that regional differences in the sensory innervation of the bladder wall may provide a structural correlation for these different sensations [45]. Furthermore, it has been demonstrated by Barrington some 80 years ago that activation of sensory fibres from different regions of the LUT contributed to different reflexes (the 7 Barrington's reflexes) [46]. The finding of different types of nerve fibres in the same region and different patterns of innervation in different regions might also provide structural correlates with Barrington's functional data.

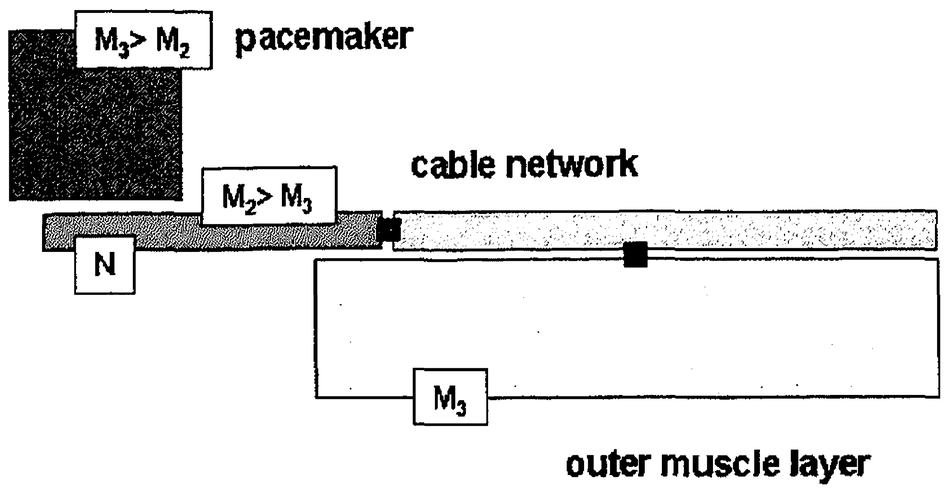

Figure 1. A schematic diagram suggesting possible mechanisms in the normal and pathological bladder to account for autonomous activity and the actions of arceaidine. The concept is proposed that there are two means to activate the detrusor: motor nerves of the postganglionic component of the parasympathetic system, and a network of interstitial cells. The motor nerves are used to activate the micturition event while the interstitial cell network is involved in the generation, modulation and distribution of activity which underlies phasic non-micturition activity (autonomous activity). The network of interstitial cells is complex and heterogeneous [34,35]. Functionally different elements can be identified: (i) a pacemaker and a distribution network. Activity in the pacemaker is driven by multiple inputs which include a cholinergic component. In the control group this mechanism involves M_3 muscarinic receptors. Also, based on data from the control group, activity within the distributing interstitial cell network is increased by muscarinic stimulation. The concept proposed is that muscarinic stimulation increases the spread of activity in the network and leads to a recruitment of muscle bundles and consequently larger increases in pressure. Activity in the interstitial cell network is conveyed to the individual muscle bundles directly by a mechanism possibly involving gap junctions. The same basic functional arrangement is present in the other groups, but the pacemaker mechanism appears to be altered and has a greater sensitivity to muscarinic agonists. The distribution of activity is also altered in the operated groups, such that it is fully activated in the unstimulated bladder. In this respect the autonomous activity in the operated groups is similar to the activity produced by the nicotinic ligand lobeline. Interestingly, excessive activity after muscarinic agonists or during exposure to adrenergic stimulation has the effect of uncoupling the network, affecting the spread of activity and so reducing the amplitude of the contractions. The hypothesis is proposed that the changes in bladder function after damage to the bladder neck are the result of changes to the network of interstitial cells. These changes affect two functions of the network, its pacemaker role and distribution of activity.

Conclusion

This thesis confirms that the SU-ICs, SM-ICs and MC-ICs appear to be distinct populations. The SU-ICs express receptors to acetylcholine (M_3), prostaglandin (EP2) and atrial naturetic peptide. M_3 receptors play a role in the autonomous contractions of the bladder. Changes in these autonomous contractions are associated with bladder dysfunction, causing a sensation of urgency during the filling phase and an increased frequency of voiding [4]. These observations are in line with the above hypothesis. This leads to the suggestion that M_3 receptors on SU-ICs are important in the activation of the phasic bladder contractions. Therefore, M_3 specific anticholinergic drugs are effective in treating urge symptoms in the OAB syndrome [28]. Thus it can be concluded that the ICs expressing the M_3 receptor play a key role in the motor sensory system.

Phasic activity occurs in the normal bladder in vivo as non-micturition activity [48]. Such activity has been suggested to play a central role in a motor/sensory system involved in the generation and regulation of bladder afferent nerve

activity and sensation. Evidence is thus accumulating to link cholinergic driven ICs with phasic activity and *in vivo* to link phasic activity with the generation of sensation [39, 49]. Thus, ICs play a key role in the motor/sensory mechanisms of the bladder.

We want to hypothesize that the increased pressure on the bladder neck and base by the increased size of the prostate inflicts a damage to the bladder neck. This damage might be due to pressure on efferent/afferent innervation running through these parts of the urinary bladder. As the data in this thesis suggest, the LP-ICs and SM-ICs constitute a network of ICs extending throughout the bladder, and these cells appear to be particularly sensitive to activation following bladder neck damage. Thus, it would be possible that an initial local activation could be rapidly propagated through this network resulting in activation of the entire network. This pathological change might be the first cause for changes in the autonomous activity. On the other hand the mechanism(s) of generating autonomous activity, controlling the pacemaker functions and facilitating the propagation throughout the bladder, are still not known. Nevertheless, the changes in network properties might subsequently trigger changes in the other cellular systems in the bladder wall resulting in the observed global pathology.

Future work

SU-ICs receive multiple inputs via the urothelial-derived signals [23]. These signals are likely to be involved in the activation or modulation of different pathways within the SU-ICs. These systems may act synergistically to produce or modulate a specific response. Alternatively, they may be antagonistic and, depending upon their level, integrated to produce a specific response. The SU-ICs may therefore be a point of integration of signals derived from the urothelium. Further research is necessary in order to establish if this indeed is the case. The signals generated by the urothelium and the receptors and pathways hypothesised in this thesis are illustrated in the cartoon below.

New drugs are being developed, such as the beta-3 adrenergic agonists. It has been suggested that these drugs may modulate afferent activity, which improves OAB symptoms, with lower risk of affecting voiding function. However the exact location where beta-3 adrenergic agonists work is not known. Further research is necessary in order to identify the location of the beta-3 receptor. We can speculate on what cell type this could be located. This receptor must be located on a cell type that plays a major role in the sensory/motor system. Since beta-3 adrenergic agonists do not alter micturition the receptors must be located on a cell type that influences autonomous activity, e.g. SU-ICs, LP-ICs or (intramural) ganglion cells. The results of our studies point to a control of the motor/sensory system in order to control sensory/afferent signalling and relieve symptoms of OAB.

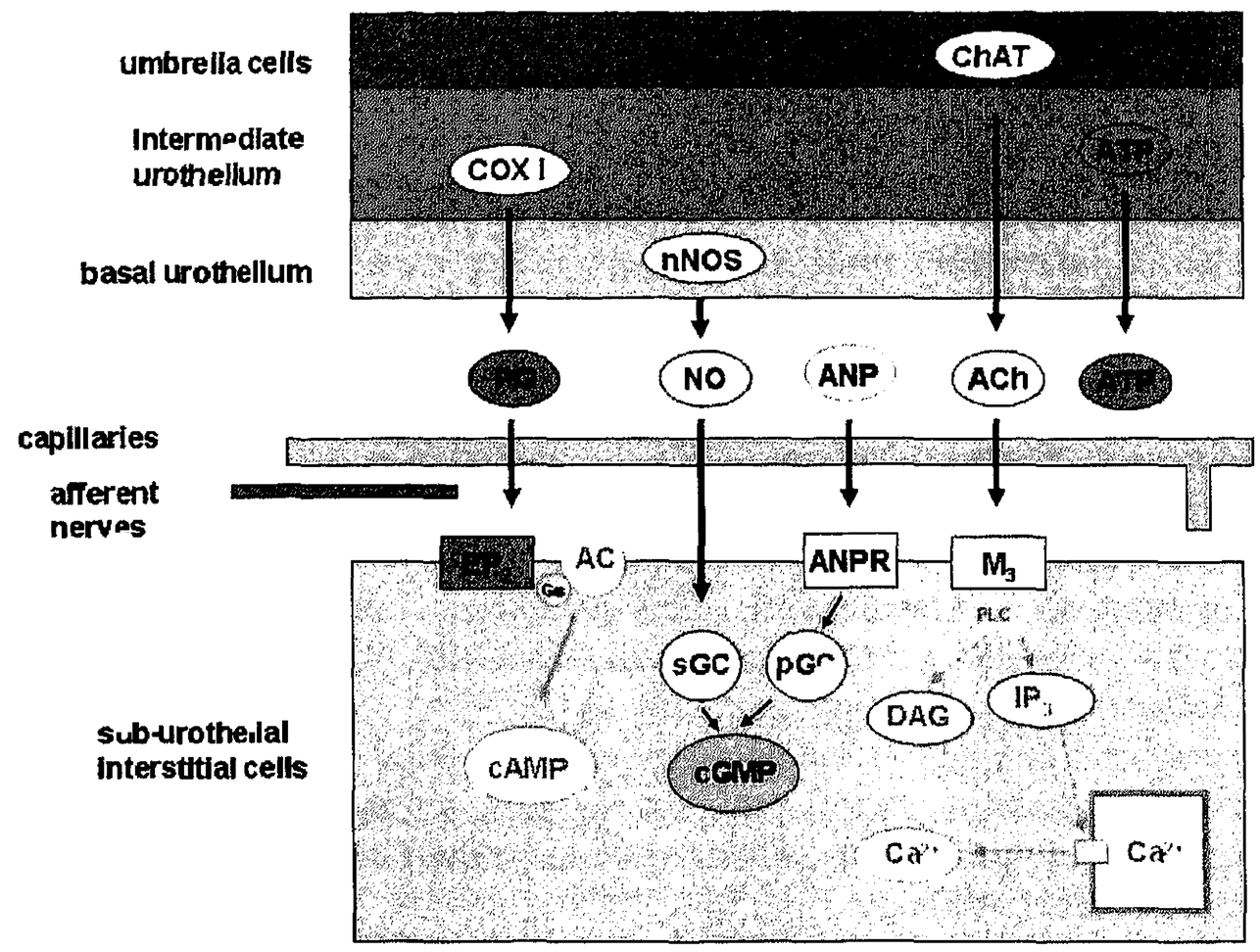

Figure 2. Cartoon illustrating the output of urothelial signals and possible interactions of these signals on the sub-urothelial interstitial cells in the guinea pig bladder. The output of the urothelium including prostaglandin (PG), nitric oxide (NO), acetylcholine (ACh), and adenosine trisphosphate (ATP) is all well documented [8, 16, 18, 31, 52, 53]. This thesis presents data that the M_3 receptors are on the SU-ICs. EP2 receptors have been demonstrated immunohistochemically and ANP receptors inferred from the actions of exogenous ANP [34, 54]. NO and ANP induce a rise in cGMP. Based on published data activation of EP2 generates a rise in cAMP via adenylate cyclase (AC) and activation of M_3 causes a rise in intracellular Ca^{2+} from intracellular stores and diacylglycerol (DAG) [36, 55]. The physiological system underpinned by these signalling elements on the SU-ICs has yet to be identified.

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Chapter 9

Summary / Samenvatting

Summary
The overactive bladder syndrome (OAB) is a common condition affecting 17% of the populations of Europe and the USA.OAB is characterised by urgency, frequency and nocturia. Nocturia means that a patient needs to go to the bathroom at least one time each night. Frequency means that a patient needs to urinate at least 8 times each day. Urgency means the extreme urge to go to urinate. The urgency can be so severe that leakage occurs and thus patients show urge incontinence. Incontinence has a great impact on daily life. People become isolated, due to the fear of incontinence in public situations.
Urgency incontinence is mostly caused by involuntary contractions of the bladder. When the pressure in the bladder supresses the sphincter pressure due to these contractions, incontinence occurs. Patients with urgency incontinence feel the need to go to the bathroom, but can not make it in time. Urge incontinence is mostly caused by a dysfunction of the bladder. Only little is know about what causes this dysfunction, how this mechanism works and how this problem can be treated. It has been shown in animal experiments and some human studies that the bladder shows autonomous contractions which might play a role in the bladder control mechanism. This control mechanism is influenced by interstitial cells (ICs) and nerve fibers. When it is known how this control mechanism works, only then specific treatment can be developed. This thesis deals predominantly with the interstitial cells in the urinary bladder: a cell type of which it is more and more recognized that they are part of the control mechanism of the bladder.

Interstitial cells of the bladder
In **chapter 2** an overview is given on what is known about the ICs of the bladder. It is known that the ICs show structural similarities with the ICs of the gut, but the immunohistochemical marker (C-kit) of the ICs of the gut is only sporadically found in the ICs of the bladder. In our studies we used vimentin as a marker for the ICs of the bladder. Vimentin is a cytoskeletal protein of fibroblasts. Since interstitial cells are a subtype of fibroblasts, it is possible to identify these cells with markers against vimentin.
In **chapter 3** describes our study on the ICs and nerves in the area between the bladder and the urethra; the bladder neck. The distribution of the sub-urothelial ICs (SU-ICs) at the location of the bladder neck is different compared to the bladder wall and the urethra. No SU-ICs were noted in the bladder neck, while in the bladder wall these cells form a layer of 1-3 cells thick directly below the urothelium. Another interesting finding was the location of clusters of ICs in the bladder-urethral junction. We noted also that nerve fibres are distributed in the urothelium of the bladder neck. Our study points out the regional differences in the bladder neck compared to the lateral wall of the bladder and the urethra. The fact that this area shows structural differences points out that this area must serve a specialised function.

In **chapter 4** the different locations of prostaglandin production in the bladder are discussed. The bladder generates spontaneous contractions, which are called autonomous contractions. These autonomous contractions play a role in the motor/sensory system of the bladder, which registers the state of filling of the bladder. Autonomous contractions are influenced by prostaglandins. For this reason it is important to know which cells are able to produce prostaglandins and thus are able to influence the autonomous contractions. One immunohistochemical study on the location of cyclo-oxygenase enzyme type I (COX I), an enzyme which is responsible for prostaglandin production, shows this enzyme in three areas of the bladder wall. These areas are: 1) the urothelial cells, 2) in ICs and 3) in unclassified cells located in the lamina propria. These unclassified cells are located in close proximity of a network of ICs which spreads from the lamina propria towards the outer muscle layer. The ICs located in the inner muscle layer contain also COX I, while the ICs located in the outer muscle do not contain COX I. This study points out that a subpopulation of ICs is able to produce prostaglandins.

In **chapter 5** the response of the IC network of the bladder to bladder outlet obstruction is described. A bladder outlet obstruction is an obstruction of the urethra, which in patients often occurs due to benign prostatic enlargement. A bladder outlet obstruction causes bladder overactivity. In our study we create a bladder outlet obstruction by placing a ring around the urethra. In sham operated animals the same procedure took place as in the obstructed bladders, but only without implanting the ring around the urethra. The structures of the sham operated and control bladders were compared with the obstructed bladders. Ubiquitin hydrolase (UBH) is an enzyme which plays a role in the ubiquitin-proteasome system. This enzyme is also known as protein PGP 9.5, which has been used as a marker for nerve cells and fibers. We found UBH in nerves of control, sham and obstructed bladders. In the control bladders mainly the nerves contained UBH, while in the sham and obstructed bladders also a subpopulation of ICs contained UBH. These ICs were located in the lamina propria (lamina propria ICs, LP-ICs), around the muscle bundles (surface muscle ICs, SM-ICs) and in the serosa (muscle coat ICs, MC-ICs). The number of ICs which contained UBH was profoundly more in the obstructed bladders compared to sham bladders. This finding suggests a relationship between UBH upregalation and the severity of bladder neck damage. Activation of UBH in the ICs points out that these cells are actively involved in tissue remodelling as a result of bladder outlet obstruction. These observations in this study point out that a subtype of ICs plays a role in the coordination of the changes in the bladder as a result of damage to the bladder neck.

In **chapter 6** the location of the muscarinic receptor type M_3 is discussed in normal bladders. M_3 receptors play a central role in the function of the bladder. Activation of M_3 receptors by acetylcholine in the bladder results in a bladder contraction. The precise location of the M_3 receptors in the bladder is not known. We describe the location of the M_3 receptor on a subpopulation of ICs.

These ICs are located directly below the urothelium (SU-ICs). There is a difference in the distribution of both the M_3 receptors and the SU-ICs in de bladder neck compared to the lateral wall. In the bladder neck there were less SU-ICs. The SU-ICs in this region did not contain the M_3 receptor, while the SU-ICs in the lateral wall did contain this receptor. The fact that a subset of the SU-ICs poses the M_3 receptor points to a further subdivision of these cells. The SU-ICs which contain the M_3 receptor will probably serve a different function compared to the SU-ICs that do not have this receptor.

Chapter 7 describes the location of the muscarinic receptor type M_3 in sham and obstructed bladders. Obstructed bladders show detrusor overactivity, which means that they show severe autonomous contractions. These contractions are influenced by acetylcholine, which works through the activation of the M_3 receptor. The M_3 receptor is located on a sub-population of nerves in the lamina propria and in the muscle layers. The M_3 receptor was also associated with a subpopulation sub-urothelial ICs, ICs around the muscle bundles (SM-ICs) and ICs inside the muscle bundles (intra-muscular ICs, IM-ICs). The obstructed bladders were hypertrophied compared to normal bladders. They also had less nerves and ganglia. There was no difference noted in the SU-ICs, while there was an increase in number of LP-ICs, SM-ICs and IM-ICs in the obstructed bladders. Thus, in obstructed bladders there is a decrease in nerves and a (possibly compensatory) increase in the number of ICs that contained the M_3 receptor. Since there is an increase in the number of M_3 receptors in the bladder a small amount of acetylcholine can cause a strong contraction; a cholinergic hypersensibilisation. This might explain why an obstructed bladder shows overactive contractions.

In **conclusion**, this thesis confirms that the SU-ICs, SM-ICs and MC-ICs are distinct sub-populations of interstitial cells. The SU-ICs express M_3 muscarinic receptors, which plays a role in the autonomous contractions of the bladder. Changes in these autonomous contractions are associated with bladder dysfunction, causing a sensation of urgency during the filling phase and an increased frequency of voiding. Thus M_3 receptors on SU-ICs are important in the activation of the phasic bladder contractions and the M_3 specific anticholinergic drugs may have an effect on these cells.

Furthermore this thesis hypothesizes that the increased pressure on the bladder neck and base by the increased size of the prostate inflicts damage to the bladder neck. This damage activates the network of LP-ICs and SM-ICs throughout the bladder. This pathological change might be the first sign of changes in the autonomous activity. However the mechanism(s) of generating autonomous activity, controlling the pacemaker functions and facilitating the propagation throughout the bladder are still not known. Nevertheless, the changes in network properties might subsequently trigger changes in the other cellular systems in the bladder wall resulting in the observed global pathology.

Further research is necessary in order to unravel the function of the ICs in the bladder. The SU-ICs receive multiple inputs via the urothelial-derived signals.

These signals are likely to be involved in activating or modulating different pathways within the SU-ICs. These systems may act synergistically to produce or modulate a specific response. The SU-ICs may therefore be a point of integration of signals derived from the urothelium.
Beta-3 adrenergic agonists are new drugs developed for the treatment of OAB. These drugs moderate afferent activity, which improves OAB symptoms. The location of the beta-3 receptor in the bladder is not known. This receptor must be located on a cell type that plays a major role in the sensory/motor system, e.g. SU-ICs, LP-ICs or (intramural) ganglion cells.

Samenvatting

Het overactieve blaassyndroom wordt gekenmerkt door urgency, frequency en nycturie. Nycturie houdt in dat iemand minimaal 1 keer per nacht moet opstaan om naar het toilet te gaan. Frequency houdt in dat iemand meer dan 8x per dag naar het toilet moet. Bij urgency bestaat er een onbedwingbare aandrang om te urineren. Dit kan zelfs zo erg zijn dat er urgency incontinentie optreedt. Incontinentie kan een grote impact hebben op het dagelijkse leven van de patiënt. Het kan zo erg zijn dat mensen er door geïsoleerd raken, omdat zij bang zijn om in het openbaar urine te verliezen.
Urgency incontinentie houdt in dat de blaas onvrijwillig samentrekt waardoor de druk in de blaas hoger wordt dan de sluitspier aankan, waardoor incontinentie ontstaat. Dit komt door een stoornis in de functie van de blaas. Onze kennis van de pathofysiologie van deze stoornis en wat eraan gedaan kan worden is tot nu toe nog zeer beperkt. De vrijwel lege blaas laat kleine kontrakties zien die hoogswaarschijnlijk een rol spelen bij de controle over de blaas. Als de exacte werking van het controle mechanisme van de blaas ontrafeld is, pas dan kunnen op rationele grondslag medicijnen ontwikkeld worden die specifiek gericht zijn tegen overactiviteit van de blaas. Het doel van deze thesis is om mee te bouwen aan het inzicht in het controle mechanisme van de blaas. Dit is hoofdzakelijk gedaan door middel van immunohistochemische studies naar een belangrijk onderdeel in dit mechanisme: de interstitiële cellen.

Interstitiële cellen van de blaas

Interstitiële cellen zijn cellen die in verschillende weefsels in het lichaam voorkomen en een scala aan functies vervullen. Zo is beschreven dat interstitiële cellen een belangrijke rol spelen in het vernieuwen van weefsel, maar ook dat zij een functie hebben in de beweging van de darm. Er zijn nogal wat aanwijzingen in de literatuur dat de interstitiële cellen van de blaas betrokken zijn bij het tot stand komen van de contracties die de blaas maakt in rusttoestand; met deze contracties 'voelen' we als het ware hoe vol onze blaas zit (autonome activiteit). Interstitiële cellen spelen een centrale rol in dit mechanisme.

In **hoofdstuk 2** wordt een overzicht gegeven van wat er tot nu toe bekend is over de interstitiële cellen van de urine blaas. Het is bekend dat zij qua structuur erg veel lijken op de interstitiële cellen van de darm, echter de immunohistochemische marker (C-kit) die gebruikt wordt om interstitiële cellen van de darm mee te identificeren wordt maar sporadisch in de interstitiële cellen van de blaas aangetroffen. Er is gezocht naar een specifieke immunohistochemische marker voor de interstitiële cellen van de blaas. In onze studies gebruiken wij vimentine als marker voor interstitiële cellen. Vimentine is een eiwit dat voorkomt in het cytoskelet van fibroblasten, en aangezien interstitiële cellen een subtype van de fibroblasten zijn, is het dus mogelijk om aan de hand van antilichamen gericht tegen het vimentine eiwit, de interstitiële cellen te bestuderen.

In **hoofdstuk 3** wordt een studie beschreven naar interstitiële cellen en de innervatie in het overgangsgebied van de blaas naar urethra; de blaashals. De distributie van de direct onder het urotheel gelegen interstitiële cellen (sub-urotheliale interstitiële cellen, SU-ICs) ter hoogte van de blaashals is volledig anders ten opzichte van de blaas. Op deze locatie bevinden zich slechts sporadisch SU-ICs, terwijl er zich in de blaas een cellaag van 1-3 cellen dik onder het urotheel bevindt. Daarnaast vindt men in de blaashals een cluster van zeer veel interstitiële cellen ter hoogte van de spierlaag. Een andere interessante bevinding van deze studie was het voorkomen van zenuwvezels in het urotheel van de blaashals. Deze studie toont dus aan dat er duidelijke regionale verschillen in de blaashals zijn ten opzichte van de blaas en urethra. Het feit dat dit gebied qua structuur totaal anders is wijst erop dat dit gebied een gespecialiseerde functie heeft.

In **hoofdstuk 4** worden de verschillende locaties van prostaglandine productie in de blaas besproken. De blaas laat in rusttoestand kleine contracties zien, die autonome contracties genoemd worden. In ons concept maken deze autonome contracties deel uit van tot een senso-motor systeem van de blaas waarmee de vullingtoestand van de blaas wordt waargenomen. Het senso-motor systeem is een mechanisme waarbij de blaas actief door een beetje samen te trekken (autonome contracties) voelt hoe ver deze gevuld is. De autonome contracties worden beïnvloed door prostaglandinen. Daarom is het belangrijk om te weten welke cellen in staat zijn om prostaglandines te produceren en dus de autonome contracties beïnvloeden. Een immunohistochemische studie naar de locatie van cyclo-oxygenase enzym type I (COX I), een enzym dat verantwoordelijk is voor de productie van prostaglandinen, toont aan dat dit enzym zich op drie plaatsen in de blaaswand bevindt. Deze drie plaatsen zijn: 1) in urotheliale cellen (cellen die de binnenbekleding van de blaaswand vormen), 2) in interstitiële cellen en 3) in nog niet nader gekarakteriseerde cellen in de lamina propria. Deze nog ongekarakteriseerde cellen liggen dichtbij het netwerk van interstitiële cellen dat zich uitbreidt van de lamina propria tot in de buitenste spierlaag. De interstitiële cellen gelegen in de binnenste spierlaag bevatten ook COX I, terwijl de interstitiële cellen gelegen in de buitenste spierlaag geen COX I bevatten.

Deze studie toont dus aan dat een subpopulatie van interstitiële cellen de mogelijkheid bezitten om prostaglandinen te produceren.
In **hoofdstuk 5** wordt beschreven hoe de subpopulaties van interstitiële cellen in de blaas reageren op een blaasuitgangsobstructie. Een blaasuitgangsobstructie is een obstructie van de plasbuis, welke bij mannelijke patiënten vaak veroorzaakt wordt door een vergroting van de prostaat. Een blaasuitgangsobstructie veroorzaakt overactiviteit van de blaas. In ons onderzoek wordt een blaasuitgangsobstructie gemaakt door een ringetje teplaatsen rondom de plasbuis. Bij een zogenaamde sham geopereerde blaas wordt dezelfde procedure uitgevoerd, alleen wordt er geen ringetje geplaatst. Een controle blaas ondergaat uiteraard geen operatie.
Ubiquitin hydrolase (UBH) is een enzym dat deel uit maakt van het ubiquitin-proteasoom systeem. Dit systeem speelt o.m. een rol in de regulatie van celgroei en differentiatie. Tevens is dit systeem aanwezig in zenuwcellen. Wij vonden UBH in zenuwvezels van controle, sham en geobstrueerde blazen. In de controle blazen bevatten voornamelijk de zenuwvezels UBH, terwijl in de sham en geobstrueerde blazen ook een subpopulatie van interstitiële cellen UBH bevatten. Deze interstitiële cellen bevonden zich in een laag onder het urotheel; de lamina propria (lamina propria interstitiële cellen, LP-ICs), rond de spierbundels (surface muscle interstitiële cellen, SM-ICs) en in een laag tegen de spierbundels aan; het serosa (muscle coat interstitiële cellen, MC-ICs). Het aantal interstitiële cellen dat UBH bevatten was beduidend meer in de geobstrueerde blazen vergeleken met de sham geopereerde blazen. Deze bevinding suggereert dat er een relatie is met de verhoogde activiteit van UBH in interstitiële cellen en de ernst van beschadiging in de blaashals. De toename van UBH in de interstitiële cellen duidt erop dat deze cellen actief betrokken zijn bij weefsel veranderingen als gevolg van blaasuitgangsobstructie. De observaties gedaan in deze studie wijzen erop dat een subtype van interstitiële cellen een rol speelt bij de coördinatie van de veranderingen in de blaas als gevolg van blaashals beschadiging.
In **hoofdstuk 6** wordt de locatie van de muscarine receptor type M_3 beschreven in de normale blaas van de cavia. Muscarine speelt een belangrijke rol in de activiteit van de blaas. Muscarine activeert M_3 receptoren, welke dus een centrale rol spelen in de functie van de blaas. Activatie van de M_3 receptoren door acetylcholine veroorzaakt een blaascontractie. De locatie van de M_3 receptoren in de blaas is tot op heden nog niet beschreven. In deze studie beschrijven wij de locatie van de M_3 receptor en identificeren we de celtypen die in het bezit zijn van deze receptor. De meest opvallende bevinding was dat de M_3 receptor geassocieerd was met de suburotheliale interstitiele cellen (SU-ICs). Er is een verschil in distributie van zowel de M_3 receptor als de SU-ICs in de blaashals ten opzichte van de laterale wand. In de blaashals waren beduidend minder SU-ICs cellen aanwezig. De SU-ICs in deze regio waren niet in het bezit van de M_3 receptor, terwijl de SU-ICs in de laterale wand wel in het bezit zijn van de M_3 receptor. Het feit dat sommige SU-ICs in het bezit zijn van de

M_3 receptor wijst op een verdere onderverdeling en verschil in functie van deze cellen.

Hoofdstuk 7 beschrijft de locatie van de muscarine receptor type M_3 beschreven in sham geopereerde blazen en blazen met een uitgangsobstructie. Obstructieve blazen vertonen overactiviteit, dat wil zeggen dat zij meer en heftigere autonome contracties vertonen. Deze contracties worden onder andere beïnvloed door acetylcholine (een stof dat muscarine receptoren beïnvloed), dat zorgt voor activatie van de M_3 receptor. De M_3 receptor is waargenomen op een sub-populatie zenuwvezels en neuronen in de lamina propria en in de spierlagen. Ook was de M_3 receptor geassocieerd met een subpopulatie van sub-urotheliale interstitiële cellen, interstitiële cellen rond de spierbundels (SM-ICs) en interstitiële cellen in de spierbundels (inta-muscular interstitiële cellen, IM-ICs). De blazen die een blaasuitgangsobstructie hadden en dus overactief waren, hadden een duidelijke toegename in blaaswand dikte ten opzichte van normale blazen. Ook hadden zij beduidend minder zenuwvezels en ganglia. Er was geen verandering waarneembaar ten aanzien van de SU-ICs, terwijl er een toename was in het aantal LP-ICs, SM-ICs en IM-ICs. Dus na een blaasuitgangsobstructie is er een afname van zenuwen en een mogelijk compensatoire toename van interstitiële cellen, welke in het bezit zijn van de M_3 receptor. Aangezien er een toename is in het aantal M_3 receptoren in de blaas, kan dus een kleinere hoeveelheid acetylcholine een krachtigere contractie teweeg brengen. Dit kan mogelijk verklaren waarom een blaas met een uitgangsobstructie als het ware overgevoelig is en overactieve contracties vertoont.

Concluderend, deze thesis bevestigd dat SU-ICs, SM-ICs en MC-ICs specifieke subpopulaties van interstitiële cellen zijn. De SU-ICs zijn in het bezit van een receptor door acetylcholine (M_3). Deze receptor speelt een rol bij de autonome contracties in de blaas. Veranderingen in deze contracties zijn geassocieerd met urgency en frequency. Dus de M_3 receptoren op de SU-ICs spelen een rol bij autonome blaascontracties en M_3 specifieke anticholinege medicatie heeft mogelijk een effect op de SU-ICs.

Deze thesis brengt de hypothese naar voren dat een verhoogde druk op de blaashals, veroorzaakt door benigne prostaat hypertrofie, resulteert in een beschadiging van de blaashals. Deze beschadiging activeert het netwerk van LP-ICs en SM-ICs in de blaas. De pathologische veranderingen in deze cellen dragen mogelijk bij aan de veranderde autonome activiteit. Het mechanisme van het ontstaan en de regulatie van de autonome activiteit is tot op heden onbekend. De opregulatie van het netwerk van interstitiële cellen kan verantwoordelijk zijn voor de veranderingen in de gehele blaas.

Verder onderzoek is nodig om de functie van interstitiële cellen in de blaas te ontrafelen. De SU-ICs ontvangen signalen vanuit het urotheel. Deze signalen hebben een functie in de activatie en modulatie van deze cellen. Dus de SU-ICs hebben een functie in de verwerking van urotheliale signalen.

Beta-3 adrenerge agonisten zijn nieuw ontwikkelde farmaca voor de behandeling van OAB. Deze farmaca beïnvloeden de afferente activiteit, waardoor een vermindering van de symptomen teweeg wordt gebracht. De locatie van de beta-3 receptor in de blaas is tot op heden niet goed bekend. Deze receptor moet gelegen zijn op een celtype welke een belangrijke rol speelt in het motor/sensor systeem van de blaas; dus op SU-ICs, LP-ICs of (intramurale) ganglion cellen.

Chapter 10

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Chapter 11

Curriculum Vitae and list of publications

Curriculum Vitae

Simone Grol was born in Nijmegen on the 1st of May 1980. She attended primary school in Berg en Terblijt (Limburg). After completing the MAVO (secondary school) in 1996 and the HAVO (college) in 1998 she started with the HBO-v (nursing college) in Heerlen. After one year she decided to switch to medicine. For this reason she followed the necessary courses at the VWO (grammer school) and started in 2001 with her studies in medicine at the university of Maastricht. As a medical student she followed an internship in India and Bangladesh. Her reresearch intership was conducted at the department of Urology in Maastricht. In October 2007 she obtained her medical degree and started her PhD research 'The interstitial cells of the bladder' at the University Hospital of Maastricht. In January 2009 she started her specialised training programme to become an urologist.

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S Grol, G van Koeveringe, P Essers, J de Vente, P van Kerrebroeck, J Gillespie

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EAU 2008 Milan

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